THE STRUCTURE AND FUNCTION OF CYTOCHROME P450 IN THE HEPATOPANCREAS OF THE FLORIDA SPINY LOBSTER, PANULIRUS ARGUS

By

SEAN MICHAEL BOYLE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This dissertation is dedicated to the memmory of

Bridgette Bernadette Phillips

ACKNOWLEDGMENTS

Many people have rendered support to me over the years. This section may prove to be a bit extensive.

I would like to first acknowledge my father, John Jude Boyle. He has a master's degree in sociology, a degree in medicine and was a Jesuit deacon. His analytical disposition and extremely strong dedication to medicine served as a constant example of qualities to be sought.

My mother, Donna Deloris Boyle, taught me lessons not so analytical in nature. She demonstrated time and time again that logic usually fails when applied to everyday life, and that love and compassion are the tools of existence. She is now in charge of a Hospice division in the mountains of Georgia. Somewhat fitting for her, I think.

My siblings also helped shape and guided me through the years. As children, my two older sisters, Michelle Davina and Melissa Renee, would play a game in which they were school teachers and my brother, Christopher David, and I, were the students. When the two sisters tired of the game, I would assume the role of teacher and subject my poor brother to hours more of schooling. I now have two younger sisters, Kelly Ann and Katie Marie. My stepmother, Donna, has given me plenty of moral support throughout.

The first professional teacher to instill within me a desire for knowledge was at the time a high school geometry teacher named Ronald Blatnick. His sense of humor coupled to the proficiency he enjoyed in the subject was the first example I had encountered which illustrated that learning could indeed be fun and rewarding. It was Ron who taught me how to play chess and encouraged me to begin programming computers. Programming skills would later greatly shape my scientific career.

Other teachers in high school were also exemplary. Mike Beistle taught world history, English, and theater. His classes were filled with compassion and impromptu interpretation of various subjects. Mike Muschamp was the principle and he taught American history. He was part judge and part teacher, but always fair and just. His example of how a person with integrity handles all forms of life's adversities, coupled with his rather strong Georgian accent, still serves as a role model for me.

While obtaining an undergraduate degree, I was taking a general biology class. One day it was announced that a professor needed a few students to help culture Bryozoans. I had no clue what such a creature was, but I went to see the professor anyway. I found Frank Maturo, Jr. I soon found that the questions he was asking about these small, colonial sessile invertebrates were fascinating. I also found that he was called "Doc". I spent most of my first 2 years of college in his lab. The single most important lesson he

taught me was that a carefully planned experiment could answer a question one has, and that exotic solutions to such questions are usually not desirable. As a brief example, he was interested in the question of whether or not a certain species of Bryozoan could self-fertilize. He showed me a proposal a graduate student had written to address this question. It contained many complex biochemical experiments. I told him that I thought it was a really "cool" proposal. He then asked if I could think of a better way. Well, I could not. He then said, "Why not put a colony in a jar, and see if more criters' show up".

One day I was on my way to visit "Doc" when I noticed a person in the closet across from Doc's lab. I said hello to him and asked him what he was doing in the closet. He told me his name was Mike Miyamoto and he was a new faculty member in the Department of Zoology. He told me the university had promised him a big laboratory, but instead gave him that closet. I welcomed him to the University of Florida. Mike did eventually get his lab and I went to work with him using my programming skills to help manage the mitochondrial DNA he was analyzing. Mike taught me to be as thorough as possible when analyzing or proofing data. He also introduced me to molecular biology.

Jon Reiskind, also a professor in zoology, helped me to realize that scientific research need not only be filled with hard work and stress, but can be viewed as a type of art. He worked with the speciation of wolf spiders. These

are beautiful animals with very strict geographical boundaries. I have fond memories of collecting specimens at night, spotting the spider's eyes with a head light.

While completing my undergraduate degree in zoology, I attended a lecture given by John Schell at the Whitney Marine Laboratory for Biomedical Research, or something to that effect. The name of the Lab has changed many times and is now just the Whitney Lab, after Mr. Whitney, the man who donated the money for the lab to be built. Mr. Whitney has passed away, but his wife visits every year during the annual review process. When I sat listening to John, I did not know that I would be spending the next 7 or 8 years at the Whitney Lab.

John was lecturing on the metabolism of benzo-a-pyrene in the Florida spiny lobster. He mentioned that the lobsters did not get cancer. This caught my attention. I applied to an undergraduate program at the Whitney lab and asked to work in John Schell's lab. I was told he actually worked for a one Margaret O. James. I looked up a couple of her papers, there were many, and I was hooked, line and sinker. I was working for Michael Corbett at the time, and he spoke very highly of Margaret James. I remember the time he took explaining what the "Respiratory Burst" was to a kid who barely knew what "WBC" meant. So, I asked him to write a letter of recommendation for me. I was accepted (in the off season) into the undergraduate research training program at

the Whitney Lab. This delayed my graduation by a year, but as it turned out, it was the right thing to do.

Arriving at the Whitney Lab, I expected to first meet Margaret. But instead, I met John Pritchard. He is a very tall, NIH scientist and immediately began explaining my project to me. I was to isolate apical membranes from the spiny lobster hepatopancreas. When he was done, he asked if I had any questions. I think I replied, "Dr. Pritchard was it?". But it was my lack of even basic cellular physiology that allowed me to first meet Bill Carr and Mike Greenberg. Both would come into the lab late at night and ask if I knew what the "hell" I was doing. They were both very kind in explaining osmosis, concentration gradients, passive and nonpassive uptake mechanisms. Eventually, I met all the faculty this way, and learned that each was approachable. I owe them all a great deal.

I met Robin Wallace also. I would eventually work for him over the course of one summer. I packed up my car and moved to St. Petersberg in order to work on the snook project. My car was stolen soon after. Dr. Wallace trained me to "Score" follicles from fish. The fish he used as an example was *Fundulus heteroclitus*. These are really nice fish because they are very small, but have huge follicles. This job was going to be easy. I was wrong. I was to work on *Centropomus undecimalis*, a huge fish, with tiny, little follicles. Robin Wallace has a breadth of knowledge that is wide: from classical music (did you know that Vivaldi was

known as the "Red Monk" because he had red hair?) to paintings (Robin paints and sells art work) to science (Robin wrote the book on Vitellogenin, several I think).

During this time, I did meet Margaret. But I had learned my lesson with John Pritchard. I was ready with pen and paper at my first meeting with the "Boss". I still have those first 5 pages of notes. It took me about a week just to work through them and prepare some questions. The answers to those questions raised more questions: a cycle that has been going on for 8 years. To date, she has not run out of answers. She has the uncanny ability to solve problems in fields that are not her specialty. She has on more than one occasion solved problems I was having in molecular biology, often with limited information. She possesses an insight and understanding about Science in general that, as far as I have seen, very few scientist achieve. I feel privileged to have been her student.

As for the other members on my committee, I know little of them on a personal level. But each was chosen because of the respect they command in their given fields. Ray Bergeron and his group are well known to both the medical and industrial fields. He is difficult to keep up with in a conversation and giving seminars with him around strikes fear in the heart of many a graduate student. But more often than not, his questions gently lead the student into deeper contemplation of a given subject.

I first became aware of Bill Buhi and his lab when I heard of some studies he was doing with a faculty member in zoology. The study dealt with a protein (oviduct secretory protein?) that he was trying to detect in alligators and pigs. Several years later, our lab would look at P450s in various species with an antibody that he and Idania Alverez helped produce. I thank Idania for her help.

I first became acquainted with Kathleen Shiverick's work via a journal article. Later, I was to take several classes she taught. Of the many courses I have taken, her courses stand out in my mind as being the most clearly taught. I admit I was anxious to learn the material. I was very happy when she agreed to be a member of my committee.

The final member of my committee is Rob Greenberg. He and a then postdoc named Clay Smith have taught me most of what I know about molecular biology. Interestingly, they are nearly opposite in technique and approach to molecular biology, in my mind. I have had the advantage to incorporate both styles and feel fairly confident in my molecular biology skills. I hope to one day reach the level of understanding both men have in not only the narrow field of molecular biology, but in Science in general.

Hank Trapido-Rosenthal, a post-doctoral fellow working in Dr. Carr's lab, was the first to teach me molecular biology at the Whitney Lab. Hank was very patient and I am very much in his debt. And a special thank you to Dave Price. He was the first person to point out that certain

lambda vectors have chiral maps. I was using the wrong enatiomer for about six months before he, quite by chance, asked me how my work was progressing. After a few minutes talking with Dave, my project began to work just fine.

Jason Li was the first graduate student I met in Margaret's group. Jason and I quickly became friends. He taught me a great deal about HPLC function and microsome preparation. I owe a great deal to Dr. Li Chung-Li. His kindness both in and out of the lab made my time as a graduate student a very positive experience. He and his wife, Gena, often fed me, and allowed me to play with their two wonderful children.

Gary LaFleur was a graduate student under the supervision of Robin Wallace. Gary always had a quietness about him and could befriend an angry rattle snake. He was always willing to help anyone who asked. This trait cost him many a long night, as he would have to catch up with his work. He is a kind soul and I am fortunate to know him and his wife, Susanna.

The other students and post-docs at the Whitney Lab were all helpful. Mike Jeziorski is a post-doc who will actually stop what he is doing and look up an answer to a question you might ask of him, if he does not already know the answer. My guilt concerning this trait eventually caused me to start asking questions of Rob instead of Mike. Rob now tells me to look it up. Steve Munger was another student who would without fail offer assistance if you asked. In fact,

he frequently offered assistance even if you did not ask. But to be honest, I don't ever recall turning down his help. Gena White, a technician, also never failed to help if called upon. Her many years of experience were quite valuable to me during my training. I have found that technicians often know more than most.

I would like to thank both Louise McDonald and Shirley Metts. Without their help over the years, I would not have a place to live nor money to spend. I would like to also thank Lynn Milstead and Jim Netherton III for their expertise in graphics and photography. The Whitney Lab would be far less than it is without these two artists. A very special thank you to Jan Kallman, our department secretary. There is nothing Jan can't do. And thanks to Nancy Rosa. She was always busy, but could find time to help. And thanks to the folks at the editorial department who read this dissertation. Thank you "MDL".

And finally, I wish to acknowledge Mr. Billy Raulerson and Mr. Bob Birkett. Mr. Raulerson is one of those people who can build just about anything. Mr. Birkett can fix anything. I have see them both do it many times. I came to know Mr. Raulerson fairly well over the years. Often we talked about science and more times than not his experimental design would be far superior to whomever's project design we were talking about. This might seem a bit strange at first, but Mr. Raulerson could approach a problem from the outside, unbiased and unaffected by what famous

groups had done before or what a protocol dictated. I learned a great deal from him, more than he will ever know.

As unbelievable as it may be, I have left out many people I wish to thank. I have edited my original acknowledgments. Those I have left out are people more involved in my personal life, but as most know, my personal life is mostly taken up by research. I thank all my friends who have tolerated my ways. Again, I have been fortunate. Finally, thank you to Ali Farakabesh. Besides being one of my closest friends, he gave me the computer I typed this manuscript on. All of my friends are that giving. I am very fortunate indeed.

And a special thanks to Mr. Lefty. His devotion, in spite of Feline Leukemia, has been an inspiration to me and everyone who knows him. He is truly a good kitty. And he is still alive.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS..... iii

TABLE OF CONTENTS..... xiii

LIST OF TABLES..... xv

LIST OF FIGURES..... xvi

KEY TO ABBREVIATIONS.....xviii

ABSTRACT..... xx

CHAPTERS

1 CYTOCHROME P450: SOME BACKGROUND INFORMATION..... 1

Introduction..... 1
Cytochrome P450 and Cytochrome P450 Reductase..... 6
Previous Characterization of Cytochrome P450 in the Spiny Lobster..... 12
A Preview..... 17

2 CROSS-REACTIVITY OF AN ANTIBODY TO SPINY LOBSTER P450 2L WITH MICROSOMES FROM OTHER SPECIES..... 18

Introduction..... 18
Materials and Methods..... 20
Results and Discussion..... 23

3 CDNA AND PROTEIN SEQUENCE OF A MAJOR FORM OF P450, CYP2L, IN THE HEPATOPANCREAS OF THE SPINY LOBSTER, PANULIRUS ARGUS..... 32

Introduction..... 32
Material and Methods..... 35
Results and Discussion..... 41

4 CATALYTIC CHARACTERIZATION OF CYP2L1 IN BACTERIA AND YEAST EXPRESSION SYSTEMS..... 57

Introduction..... 57
Materials and Methods..... 61
Results..... 73
Discussion..... 79

TABLE OF CONTENTS, CONTINUED

5 SUMMARY OF RESULTS..... 89

REFERENCES..... 93

BIOGRAPHICAL SKETCH..... 105

LIST OF TABLES

Table page

1.1 Some CYP families and their model substrates..... 3

2.1 Classification and P450 Contents of Hepatic Microsomal Preparation of the Species Studied..... 25

3.1 The N-terminal Amino Acid Sequences in a P450-containing fraction Isolated from Spiny Lobster Hepatopancreas Microsomes..... 43

3.2 Sequence of some of the Primers Used to Obtain the cDNA Clones..... 45

4.1 In vitro Steroid metabolism in crustaceans species.. 58

4.2 Monooxygenase Activity of Spiny Lobster Cytochrome P450 Fractions in the Presence of NADPH and NADPH Cytochrome Reductase from Rat Liver..... 60

4.3 Primer Sequences Used in this Study..... 63

4.4 Expression Vectors Used in this Study and their Attributes..... 66

LIST OF FIGURES

Figure page

1.1 An example of a cytochrome P450 difference spectra... 8

1.2 Proposed reaction mechanism for P450 mediated oxygen activation and oxygenation of a substrate...10

1.3 The anatomy of the Florida spiny lobster, Panulirus argus.....13

1.4 Cross-section of the spiny lobster hepatopancreas....15

2.1 Immunoreactivity of microsomes from invertebrate and vertebrate species with anti-CYP2L antibodies generated in rabbit.....25

2.2 Composite picture of various Western blots done with invertebrate and vertebrate microsomal fractions...28

2.3 Twenty micrometer cryo-sections of spiny lobster hepatopancreas.....31

3.1 SDS-PAGE of a spiny lobster P450-containing fraction stained with Coomassie blue.....42

3.2 Cloning strategy showing the clones used to meld together a full-length cDNA sequence.....47

3.3 Nucleotide and conceptualized protein sequence of the spiny lobster cytochrome P450, CYP2L.....48

3.4 Hydropathy plots of the rat CYP2B1, rat CYP2B2, rat CYP2D4 and CYP2L.....50

LIST OF FIGURES, CONTINUED

3.5 Comparison of the deduced amino acid of CYP2L with that of rat CYPs 2B1, 2B2, 2B4 and 2D4.....52

3.6 Northern blot total RNA isolated from the hepatopancreas of the spiny lobster.....55

3.7 RT-PCR of total RNA isolated from the spiny lobster hepatopancreas.....56

4.1 The oligonucleotide sequence of expression primers MJ25, MJ24, and BRN1.....64

4.2 SDS-PAGE of induced bacterial cells (BL21) expressing cytochrome P450 2L1 from the expression vector pET28a.....74

4.3 Western blot of total cell lysate from BL21 bacterial cells expressing the pET28a construct induced with 0.4 mM IPTG.....75

4.4 SDS-PAGE of pET28a derived cytochrome P450 2L1 expressed in bacterial cells (BL21) and purified using metal chelation chromatography.....76

4.5 Western blot of microsomes from yeast expressing the cytochrome P450 2L1 insert.....77

4.6 TLC separation of progesterone and testosterone metabolites produced by expressed cytochrome P450 2L1.....84

4.7 TLC separation of progesterone and testosterone metabolites produced by expressed cytochrome P450 2L1.....87

LIST OF ABBREVIATIONS

cDNA	complementary or copy DNA
CO	carbon monoxide
CsCl	cesium chloride
CYP	cytochrome P450
Da	dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DI	sterile deionized water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
g	gram
h	hour
i.p.	intraperitoneal
K^+	potassium
KCl	potassium chloride
kb	kilobase
kD	kilodalton
kg	kilogram
M	molar
MeOH	methanol
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
M_r	molecular mass
Na^+	sodium
NaCl	sodium chloride
β-NAD^+	beta nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nmole	nanomole
P450	cytochrome P450
PAGE	polyacylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid

SDS	sodium dodecyl sulfate
SRS	substrate recognition site
Taq	*Thermus aquaticus*
TBS	tris-buffered saline
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TLC	thin layer chromatography
TRIS	Tris[hydroxymethy]aminomethane
Tween-20	polyoxyethylene-20-sorbitan
µci	microcurie
µg	microgram
µl	microliter
µm	micrometer
v	volume
w	weight
YNB	yeast nitrogen base

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE STRUCTURE AND FUNCTION OF CYTOCHROME P450 IN THE HEPATOPANCREAS OF THE FLORIDA SPINY LOBSTER, PANULIRUS ARGUS

By

Sean Michael Boyle

May, 1997

Chairperson: Margaret O. James
Major Department: Medicinal Chemistry

Cytochrome P450s are a superfamily of enzymes which participate in Phase I biotransformation reactions within a cell. These monooxygenase enzymes are found in a variety of plant and animal species, including the Florida spiny lobster, *Panulirus argus*.

Using partially purified cytochrome P450 from the spiny lobster hepatopancreas, polyclonal antibodies were obtained from rabbit sera. The antibodies cross-reacted strongly with cytochrome P450 from the spiny lobster hepatopancreas. Cytochrome P450s from other species were examined for immunoreactivity with the spiny lobster anti-P450 antibodies. Cross-reactivity was detected with the slipper lobster, but not the American lobster or blue crab. The killifish, among others, yielded strongly immunoreactive proteins. In addition, phenobarbital-treated rats also cross-reacted with the spiny lobster antibodies.

The cDNA encoding an isoform of this enzyme found in the hepatopancreas of the spiny lobster was isolated from a

cDNA library made from this tissue. This novel cytochrome P450 enzymes was designated as cytochrome P450 2L1. The deduced protein shared 35% identity with rat isoforms in the 2B family. Cytochrome P450 2L1 contains amino acids that are invariant in all known cytochrome P450s and has the highly conserved heme-binding domain.

Cytochrome P450 2L1 was expressed in the methylotrophic yeast, *Pichia pastoris*. Whole cell and microsomal fractions from yeast that expressed cytochrome P450 2L1 were catalytically active with radiolabeled testosterone and progesterone in an NADPH-dependent manner.

The major finding reported within this dissertation is the cDNA sequence of a novel cytochrome P450 isolated from the Florida spiny lobster. This cytochrome P450 represents a new subfamily, and shares structural features with cytochrome P450s found in the cytochrome P450 gene 2 family.

CHAPTER 1
CYTOCHROME P450: SOME BACKGROUND INFORMATION

Introduction

Cytochrome P450s are monooxygenases capable of oxidizing a wide variety of endogenous and exogenous compounds (Gibson and Skett, 1986). Cytochrome P450s comprise a superfamily of enzymes which are distributed in microorganisms, plants, and animals. The endogenous functions of P450s are varied. For example, in microorganisms like *Pseudomonas putida*, cytochrome P450 enables the organism to use camphor as a carbon source (Takemori et al., 1993). In plants, some cytochrome P450s are involved in the metabolism of hormones, leading to the ripening of fruit, such as in the avocado (Stegeman and Hahn, 1994). In animals, mitochondrial P450s are involved in steroid metabolism, such as the synthesis of estrogen in humans (Stegeman and Hahn, 1994). When an exogenous compound (a xenobiotic) enters into an organism, cytochrome P450s are the primary enzymes which modify the compound in order to facilitate excretion.

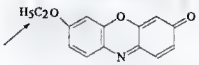
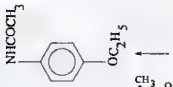
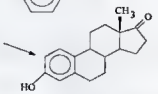
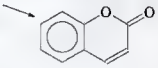
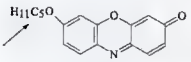
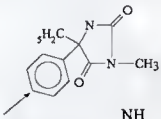
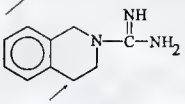
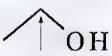
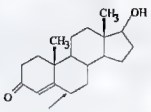
Cytochrome P450 was first discovered in 1955 at the University of Pennsylvania by Drs. G. R. Williams and M. Klingenberg (Omura, 1993). The two researchers independently

noted that when rat liver microsomes were bubbled with carbon monoxide and then reduced with nicotinamide adenine dinucleotide phosphate (NADPH), a peak at 450 nm was observed. In 1962, Drs. T. Omura and R. Sato at Osaka University confirmed that the enzyme contained a b-type cytochrome and named the protein "P-450" for "a pigment with absorption at 450 nanometers".

Cytochrome P450s are membrane-bound in eukaryotic organisms and are found in the endoplasmic reticulum (or "microsomes" when the endoplasmic reticulum is disrupted and forms aggregates) and in the mitochondria (Black, 1992). In prokaryotic organisms, cytochrome P450s are soluble and are found in the cytoplasm.

Cytochrome P450s are assigned to one of 74 gene families based on the amino acid identity of the cytochrome P450 in question to all other known cytochrome P450 amino acid sequences (Nelson et al., 1993). If the apoprotein is greater than 40% identical on the amino acid level to cytochrome P450 apoproteins of a particular gene family, then that cytochrome P450 is placed into that same gene family. If the apoprotein is greater than 55% identical on the amino acid level to cytochrome P450 apoproteins of a particular gene sub-family, then that cytochrome P450 is placed into that same gene subfamily. Table 1.1 lists a few cytochrome P450 families and model substrates that are metabolized by certain cytochrome P450 isoforms. The substrates listed in table 1.1 are substrates that are

Table 1.1 Some CYP families and their model substrates.

CYP	Model Substrate	Structure
1A1	Ethoxyresorufin	
1A2	Phenacetin	
1B1	Estrone	
2As	Coumarin	
2Bs	Pentoxyresorufin	
2Cs	Mephenytoin	
2Ds	Debrisoquine	
2E1	Ethanol	
3As	Testosterone	
4As	Lauric acid	

Arrows indicate the position of monooxygenation by cytochrome P450 enzymes.

characteristically metabolized by a particular cytochrome P450 enzyme or cytochrome P450 enzymes within that subfamily, but does not exclude the possibility that these same substrates are metabolized by cytochrome P450 enzymes in other sub-families and families. In fact, cytochrome P450s have a broad substrate preferences. An important function of cytochrome P450 in families 1 to 4 is the monooxygenation of exogenous compounds (xenobiotics).

The genes that encode mammalian cytochrome P450 enzymes can be induced by various compounds. Benzo-a-pyrene or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), for example, causes the increased transcription of the cytochrome P450 1A1 gene (Fujii-Kuriyama, 1993). Phenobarbital causes increased transcription of cytochrome P450 2B genes (Fujii-Kuriyama, 1993). Other compounds may stabilize existing mRNA levels, as is thought for cytochrome P450 2E1 induction by EtOH (Fujii-Kuriyama, 1993).

Cytochrome P450s have been detected in most tissues (in humans, erythrocytes and striated muscle lack cytochrome P450). Cytochrome P450s exist as two general classes: a group of enzymes localized in particular tissues involved typically in steroidogenesis and a group involved in the metabolism of xenobiotics (Gonzalez, 1992). Xenobiotics are defined as molecules that are not utilized by the body for energy or the normal regulation of a physiological process. Cytochrome P450s are important in determining the duration of action and toxicity of various drugs, such as

acetaminophen. How long a xenobiotic remains in the body is often determined by cytochrome P450 metabolism, especially if the xenobiotic is lipophilic.

The liver is the organ that generally contains the highest levels of cytochrome P450 in most species. Buhler et al. (1992) demonstrated that the rat liver regionally expresses various forms of cytochrome P450. Anundi et al. (1993) speculated (and demonstrated in the rat liver) that acetaminophen toxicity may be centrilobulary restricted due to localized expression of cytochrome P450 2E1. Others have further defined the regional expression of cytochrome P450s 1A1/2, 2E1, 2B1/2, and 3A1/2 (Oinonen et al., 1996, 1994; Anundi et al., 1993). Interestingly, cytochrome P450s 2C11/12 are not zone-restricted.

In the human brain, cytochrome P450s are important in the detoxification of xenobiotics, including psychoactive drugs, such as serotonin (5-hydroxytryptamine) uptake blockers (Baumann and Rochat, 1995). It has been reported that mutations in the cytochrome P450 2D6 gene have been associated with Alzheimer's disease (Saitoh et al., 1995). In microsomal fractions from rat brain, cytochrome P450s 2C7, 2C11, 2E1, 4A3, 4A8 and a 2D have been identified by N-terminal microsequencing (Warner et al., 1994) and low levels of cytochrome P450 17 protein expression have been detected (Sanne and Kreuger, 1995).

Cytochrome P450s in the eye (Stoltz et al.,1994), kidney (Ma et al., 1993), arteries (Escalante et al., 1993),

skin (Toda et al., 1994) and muscle (Pereira et al., 1994) are important in the metabolism of arachadonic acid into physiologically active metabolites known as eicosanoids (Coon et al., 1992). Compounds derived from arachadonic acid, such as 12-hydroxyeicosatetraenoic acid, lower intraocular pressure in the eye and modulate activity of the Na^+/K^+ ATPase in the eye, kidney and muscle.

Cytochrome P450s are found in both breast and ovarian tissues, where they mediate estrogen biosynthesis. Estrogen levels increase in the follicle as the follicle develops, and decrease at ovulation (Tilly et al., 1992). Both estrogen, and cytochrome P450 19 protein (the cytochrome P450 enzyme that catalyzes the conversion of testosterone to 17β-estradiol), are elevated in breast tissues from breast cancer patients (Brodie, 1993).

Cytochrome P450 and Cytochrome P450 Reductase

Cytochrome P450 is a phase I enzyme, a member of a large group of diverse enzymes involved in the first steps of xenobiotic metabolism. Cytochrome P450 utilizes molecular oxygen and reducing equivalents derived from NADPH in order to insert an oxygen atom into a substrate (Guengerich and McDonald, 1990). Cytochrome P450 is a monomer and has a molecular mass of approximately 45-60 kDa. The enzyme is anchored (Brown and Black, 1989, Black, 1992) to the

endoplasmic reticulum and contains a non-covalently bound iron protoporphyrin IX prosthetic group.

When the cytochrome P450 enzyme is reduced with a reducing agent such as NADPH or diothionite, and then complexed with CO, a maximal absorbance at 450 nm is observed (Omura and Sato, 1964, see figure 1.1). It is this characteristic of these monooxygenase enzymes that accounts for the name "cytochrome P450".

Figure 1.2 outlines the reaction mechanism between enzyme, substrate and oxygen. Cytochrome P450 binds both molecular oxygen and substrate and requires electrons (reducing equivalents) from cytochrome P450 reductase. It is thought that when the substrate binds (step 2) to cytochrome P450, a conformational change occurs within the enzyme, allowing the first electron donation (step 3)from the reductase (figure 1.2). Molecular oxygen then binds to the reduced enzyme complex (step 4). Cytochrome P450 reductase is an oxidoreductase (molecular mass around 78 kDa) and is found in close association with the cytochrome P450. The reductase accepts 2 electrons from NADPH (in the form of reducing equivalents) and donates 2 electrons sequentially to the cytochrome P450 (Smith et al., 1994). Cytochrome P450 reductase contains both flavin adenine dinucleotide and flavin mononucleotide (FAD and FMN respectively) and uses these flavins in the oxidized and reduced form (quinone and semiquinone states) to pass single electrons to cytochrome P450. The second electron may also

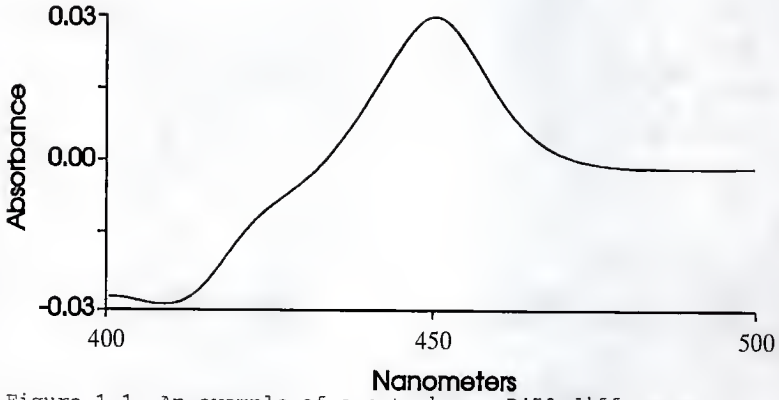


Figure 1.1. An example of a cytochrome P450 difference spectra. Spiny lobster microsomes (solubilized in 0.5% cholic acid) were diluted to about 1 mg/ml and bubbled with CO. A portion of the sample was then reduced with sodium dithionite, and the other portion was used as a reference solution. The spectrum was recorded from 500 to 400 nm. This sample has a cytochrome P450 content of 1.28 nmol P450/mg protein.

be donated by cytochrome b_5 in some instances (step 5). Oxygen scission occurs (step 5), with loss of one of the oxygen atoms to water.

Cytochrome P450s introduce oxygen into alkanes, heteroatom-containing alkanes or π-bonded systems (step 6) by variations on a radical type mechanism (Guengerich and McDonald, 1990 and Koymans et al., 1993). In each case, a radical is formed (on the substrate) either by hydrogen abstraction or electron transfer followed by radical recombination with a hydroxyl radical formed at the heme site. Loss of a second hydrogen from the substrate would form an unsaturated compound (Guengerich and McDonald, 1990). The cytochrome P450 enzymes is regenerated to the ferric state when the hydroxylated product is released (step 1). Cytochrome P450 reductase and oxygen can be replaced with an organic peroxide to complete the reaction (by going to point 6 directly from point 2).

This dissertation concerns the CYP enzyme systems in crustacea and describes the use of the Florida spiny lobster, *Panulirus argus*, as an animal model. The spiny lobster is a commercially important species in Florida due to consumer demand of this sea food. Over 4 million Kg of spiny lobster were harvested from the Florida Keys in 1992. The shellfish industry represents an important fraction of South Florida's economy. The spiny lobster offers an animal model whose anatomy (figure 1.3) and presumably enzyme systems are evolutionary divergent from our own and from

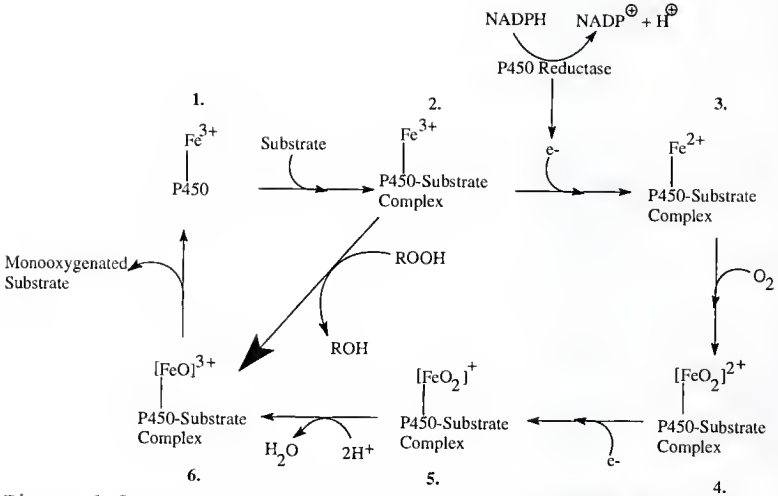


Figure 1.2. Proposed reaction mechanism for P450 mediated oxygen activation and oxygenation of a substrate. ROOH, an organic peroxide, can be used as an oxygen donor to cytochrome P450.

other common animal models such as the rat or mouse. For example, in mammals, certain cytochrome P450 genes are inducible or upregulated by chemicals such as 3-methylcholanthrene (cytochrome P450s in the 1A gene subfamily) and phenobarbital (cytochrome P450s in the 2A, 2B and 2C gene subfamilies), producing large amounts of the particular cytochrome P450 protein. Fish do not undergo gene upregulation in response to phenobarbital, but do respond to 3-methylcholanthrene by upregulating cytochrome P450 enzymes in the 1A gene family. Crustacea do not respond to either 3-methylcholanthrene (James, 1989) or phenobarbital (Stegeman and Hahn, 1994).

Lobsters have been used as models in several studies. FMRFamide-like peptides have been isolated from the American lobster (Worden et al., 1995) and have been shown to potentiate transmitter release in the nerve terminals to muscle and cause muscle contraction directly. Crustaceans have a primitive immune system, consisting of cellular and humoral responses (Takahashi et al., 1995). Spiny lobsters have been shown, like salmon and mole rats, to use polarity as a means of navigation (Lohmann et al., 1995).

An intriguing reason to study the enzyme systems of the spiny lobster is that the lobster is apparently resistance to carcinogenesis. It is believed that crustacea do not undergo carcinogenesis (Mix, 1986). An understanding of the metabolic pathways, especially those leading to reactive

intermediates in both sensitive and resistant species, may yield more insight into the mechanism of carcinogenesis.

Previous Characterization of P450 in the Spiny Lobster

The James group have characterized both phase I and II systems in both the spiny lobster (James, 1990, Schell and James, 1989) and in the American lobster (James et al., 1989, Li and James, 1993).

The hepatopancreas is a fatty, digestive gland found in all crustacea and consists of blind-ending tubules (figure 1.4). The primary function of the hepatopancreas is secretion of digestive enzymes into the stomach and the subsequent uptake of nutrients (Gibson and Barker,1979).

The hepatopancreas of the spiny lobster contains cytochrome P450 in amounts comparable to those found in rat liver (~ 1 nmole P450/mg microsomal protein, James and Little, 1980). The major site of xenobiotic biotransformation in the spiny lobster is the hepatopancreas, although cytochrome P450 has been detected in the antennal gland and in the nose of this animal.

Cytochrome P450 has been partially purified from the hepatopancreas of the spiny lobster (James,1990). Microsomes prepared from the spiny lobster hepatopancreas contain high levels of cytochrome P450. Solubilization of the microsomes produces an enriched cytochrome P450 fraction termed the M1 fraction or "red fraction" (James and Little, 1980). The red

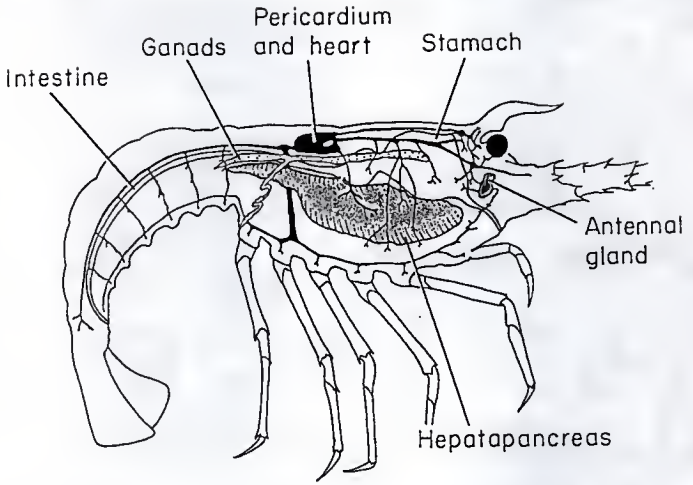


Figure 1.3. The anatomy of the Florida spiny lobster, *Panulirus argus*. The hepatopancreas is an organ analogous to the mammalian liver and contains large amounts of cytochrome P450 (~ 1 nmol cytochrome P450/ mg microsomal protein).

fraction can be resolved into partially purified P450s using anion exchange, hydrophobic interaction and absorption chromatography (James, 1990).

Reconstitution experiments using cytochrome P450 isolated from the hepatopancreas from the spiny lobster, and substrates such as benzphetamine, progesterone, testosterone and benzo-a-pyrene, demonstrated that the spiny lobster cytochrome P450 is able to metabolize a diverse group of substrates (James, 1989, James, 1990). Little activity was reported with ethoxy- or pentoxy- resorufin, substrates characteristically metabolized by cytochrome P450 enzymes in the gene subfamilies 1A and 2B, or with ecdysone, the molting hormone in spiny lobsters. (James, 1990).

The above studies were done using cytochrome P450 reductase from rat liver microsomes. To date, cytochrome P450 reductase from spiny lobster hepatopancreas microsomes has not been purified. Low cytochrome c reductase activity has been detected (James and Little, 1980) in spiny lobster hepatopancreas microsomes and hepatopancreas cytosol. The ratio of cytochrome P450 to cytochrome P450 reductase in mammals is in the range of 10:1 to 100:1; therefore concentrations of cytochrome P450 reductase in the spiny lobster may be very low. However, other artificial pathways can be used to supply single electrons to cytochrome P450 (for example, the use of peroxides), so it is possible the spiny lobster uses a novel pathway to pass electrons to cytochrome P450 *in vivo*. Cumene hydroperoxide-dependent

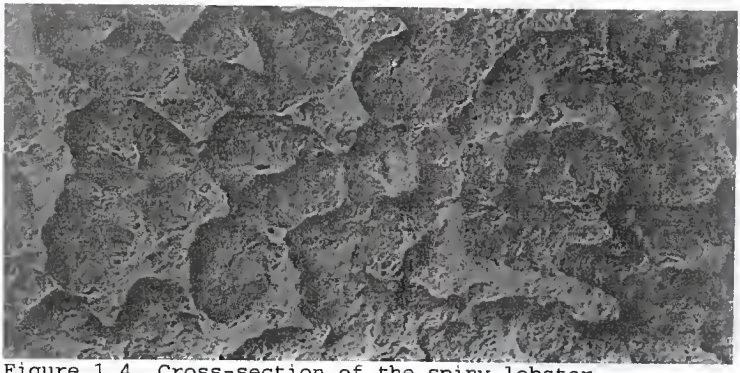


Figure 1.4. Cross-section of the spiny lobster hepatopancreas. Tissues were frozen and 20 mm sections cut. The circular structures are the blind-ending tubules.

monooxygenation of several substrates was similar to NADPH-dependent activity in M1 fractions (James, 1984). For example, mollusks may use a NADPH-independent cytochrome P450 pathway (Livingstone et al., 1989) to oxidize xenobiotics. Another plausible reason for failure to isolate cytochrome P450 reductase from the spiny lobster is that it may have been degraded by digestive enzymes and bile salts liberated during the isolation procedure (James, 1990).

Studies addressing the apparent resistance of spiny lobster to chemical carcinogenesis have yielded some insight into this phenomenon (James et al., 1992). Spiny lobsters dosed with increasing amounts of the carcinogen benzo-a-pyrene indicated a dose-dependency in DNA adduct formation. Benzo-a-pyrene is metabolized into a reactive intermediate which covalently binds to DNA. Interestingly, when the southern flounder (*Paralichthys lethostigma*, a carcinogen sensitive species) was fed hepatopancreas from a spiny lobster dosed with radiolabeled benzo-a-pyrene, DNA adducts were formed in the liver and the intestinal DNA of the fish (James et al., 1991). These studies suggest trophic transfer is a potential threat to consumers of this species and serve to reinforce the use of the spiny lobster as a model system for studying questions concerning carcinogenesis and transfer of carcinogenic chemicals among species.

A Preview

In the following chapters, studies concerning the structure and function of cytochrome P450 in the Florida spiny lobster will be presented.

An antibody to spiny lobster cytochrome P450 has been generated and used to screen microsomal fractions from other invertebrate and vertebrate animals. The spiny lobster cytochrome P450s seem to share epitopes with some invertebrate and vertebrate species. There is preliminary evidence that the cytochrome P450s in the spiny lobster hepatopancreas may be localized to certain cell types in the hepatopancreas.

The primary structure of one isoform of cytochrome P450, cytochrome P450 2L1, has been determined and is most similar to known cytochrome P450s found in rats. Hydropathy plots reveal overall similarity in predicted secondary structure as well. Northern blot and RT-PCR analysis indicate that a possible alternatively spliced form of the mRNA for cytochrome P450 2L1 may be present in the hepatopancreas.

Cytochrome P450 2L1 was inserted into a vector and transfected into the yeast *Pichia pastoris*. Upon incubation with radiolabeled testosterone and progesterone, both intact yeast and yeast microsomes yielded a 16α-hydroxylation product.

CHAPTER 2
CROSS-REACTIVITY OF AN ANTIBODY TO SPINY LOBSTER P450 2L WITH MICROSOMES FROM OTHER SPECIES

Introduction

Individual members of the superfamily of cytochrome P450 enzymes catalyze the oxidation of a wide variety of endogenous and xenobiotic substrates (Omura et al., 1993; Ortiz de Montellano; 1986; Ruckpaul and Rein, 1984). Members of one or more of the cytochrome P450 families have been found in diverse species of both plant and animal kingdoms, and the cytochrome P450 enzyme system is thought to be widespread (Nelson et al., 1993). While the gene and protein sequences of many mammalian cytochrome P450s are known (Nelson et al., 1993), much less is known about cytochrome P450s in fish and aquatic invertebrate species.

Fish cytochrome P450s have been cloned from rainbow trout (*Oncorhynchus mykiss* , cytochrome P450s 1A1, 2K1, 11A, 17 and 19) and plaice (*Pleuronectes platessi*, cytochrome P450 1A1; Stegeman and Hahn, 1994). We recently cloned a cytochrome P450 (cytochrome P450 2L) from the Florida spiny lobster, *Panulirus argus* (James et al., 1993). The only other cytochrome P450 sequence that has been cloned from an aquatic invertebrate to date is that of the pond snail (*Lymnea stagnalis*, cytochrome P450 10, Nelson et. al.,

1993). Of the other invertebrate species (Nelson et al., 1993), cytochrome P450 have been cloned from the house fly (*Musca domestica*, cytochrome P450 6A1), fruit fly (*Drosophila melanogaster*, cytochrome P450s 4D1, 4E1 and 6A2), butterfly (*Papilio polyxenes*, cytochrome P450 6B1) and cockroach (*Blaberus discoidalis*, cytochrome P450 6C1). The spiny lobster cytochrome P450 2L is the first complete member of the cytochrome P450 2 gene family from an invertebrate, and to date the second non-mammalian cytochrome P450 2 gene family form.

In mammalian species, the cytochrome P450 2 gene family is very important for monooxygenation of a wide range of structurally diverse xenobiotics and endogenous substrates (Omura et al., 1993; Ortiz de Montellano; 1986; Ruckpaul and Rein, 1984; Nelson et al., 1993). Although sequence identity of the spiny lobster cytochrome P450 2L form with other cytochrome P450s was low, certain regions of the primary sequence showed very high similarity to other 2 family members (James et al., 1996), suggesting that there may be epitopes in common. Few studies have investigated the cross-reactivity of invertebrate cytochrome P450s with vertebrate cytochrome P450 antibodies. One study found cross-reactivity of an anti-scup cytochrome P450 1A antibody to microsomal fractions of the sea star, *Asterias rubens* (den Besten et al., 1993). Another study found that microsomes made from the mid-gut gland of the chiton *Cryptochiton stelleri* cross-reacted with an antibody to

rainbow trout cytochrome P450s 2K1 and 1A1 (Schlenk and Buhler, 1989).

The objective of the present study was to investigate whether an antibody to a microsomal cytochrome P450 isolated from the hepatopancreas of the Florida spiny lobster would cross-react with microsomal fractions isolated from hepatopancreas and liver of other invertebrate and vertebrate species.

Materials and Methods

Antibody Preparation

Partially purified cytochrome P450 (11.5 nmol spectrally measured cytochrome P450/mg protein) was isolated from microsomes prepared from hepatopancreas of the Florida spiny lobster by ion-exchange, hydrophobic and absorptive chromatography (James, 1990). Samples were subjected to SDS-PAGE in one dimension (Laemmli, 1970). The major band from SDS-PAGE (52.5 kD apparent molecular mass) was detected with Coomassie blue dye and excised. Each gel slice contained about 3.0 µg of cytochrome P450 as determined by difference spectra (see below). Six micrograms of cytochrome P450 were homogenized in 1 ml of a 50% Freunds complete adjuvant-saline solution. The homogenate was then sheared with a 19 gauge needle. Pre-immunization serum was obtained from a pathogen free, New Zealand White rabbit 2 weeks earlier. The

rabbit was immunized with four 0.25 ml injections along the back. The rabbit received boosters of 6 µg of cytochrome P450 in 1 ml of 50% Freunds incomplete adjuvant-saline every 2 weeks. A total of seven immunizations were given, with detectable titers (as detected by Western blotting) beginning after the third injection.

Microsome Preparation

The fish and invertebrates used in these studies (see table 2.1) were locally caught, adult feral species of either sex, with the exception of the channel catfish. The channel catfish (*Ictaluris punctatus*) were obtained from the LSU aquaculture facility and were 800+/-100 g body weight. The rats were male, Sprague-Dawley strain, and were 200+/-20 g. The phenobarbital-induced rats were pretreated with 80 mg phenobarbital/kg i.p. for 4 days before sacrifice on the fifth day. Microsomes were prepared as described previously (James, 1990). Briefly, tissues were removed from the animal and homogenized in 0.05 M potassium phosphate (pH 7.4), 1.15% KCl, 0.1 mM EDTA, 0.2 mM PMSF. The homogenate was centrifuged at 13,000g and the supernatant centrifuged at 176,000g to pellet the microsomes. Solubilized microsomes (M1 fractions) were isolated from the invertebrates by stirring the microsomes at 4°C for 1 h in buffer containing 0.01 M potassium phosphate (pH 7.6), 20% v/v glycerol, 0.5% w/v sodium cholate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.2

mM PMSF (1 ml buffer/g wet weight hepatopancreas) and centrifuging at 176,000g for 90 min. Protein contents were determined by the method of Lowry et al. (1951). Concentrations of cytochrome P450 in the samples were determined by CO difference spectra (see table 2.1) (Estabrook et al., 1972).

Western Blots

Samples of microsomal protein, 200 µg, were subjected to SDS-PAGE on 4%-8.5% discontinuous gels in a Protean II apparatus (BioRad). Proteins were electro-blotted onto nitrocellulose using a Tris-glycine-methanol buffer system (25 mM Tris base, 192 mM glycine, 20% v/v methanol). After the transfer, the membranes were blocked in 3% gelatin-TBS (Tris-buffered saline, 20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h. The primary antibody (1:200 in 1% gelatin-TBS-0.05% Tween-20) was applied for 2 h and secondary antibody (Biorad goat-anti-rabbit alkaline phosphatase, 1:3000) was applied for 1 h. Detection was by color development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BioRad).

Immunocytochemistry

Hepatopancreas was fixed overnight in Zamboni's fixative (2% Paraformaldehyde and 0.15% picric acid in 0.1 M potassium phosphate, pH 7.4). Tissues were then subjected to

increasing concentrations (0,10,20 and 30% (w/v)) of sucrose (w/v) in PBS (phosphate buffered saline, 20 mM potassium phosphate, pH 7.4, 500 mM NaCl) for 2 h at each concentration, allowing the tissues to remain in 30% sucrose in PBS overnight. Tissues were frozen in O.C.T compound (10% (v/v) polyvinyl alcohol and 4% (v/v) polyethylene glycol, Miles Inc.) and sectioned (20 μm) on a cryostat. Sections were blocked in 1.0% (w/v) normal goat serum for 30 min. Sections were washed once for 15 min in PBS and incubated for 1 h with the primary antibody (1:50 in PBS/1% normal goat serum). Sections were washed (2 X 15 min) in PBS and the secondary antibody (goat-anti-rabbit fluorescein isothiocyanate, 1:50) applied for 1 hr. Slides were viewed with a fluorescent microscope.

Results and Discussion

Studies of the immunological relationships between cytochrome P450s in aquatic species have mostly been done in fish. We isolated microsomes from representative species in both the cartilaginous and bony fish classes and in the class crustacea. Table 2.1 list the systematics of the species we screened with the anti-spiny lobster cytochrome P450 antibody.

As expected, anti-spiny lobster cytochrome P450 antibody consistently cross-reacted with microsomal fractions, solubilized fractions (M1) and partially purified

cytochrome P450 from the hepatopancreas of the spiny lobster. Three bands were usually detected, at high molecular mass (not shown), at 52.5 kD (figure 2.1 and figure 2.2) and at 30 kD (not shown). We have Northern blot and RT-PCR evidence for what appears to be a splice variant of about 1.5 kb of cytochrome P450 2L (James et al., in preparation), and the 30 kD immunoreactive band may either represent the translated product of this cytochrome P450 2L truncated message, or perhaps is a breakdown product of cytochrome P450. Under conditions used in this study, cytochrome P450 2L can be detected at 0.05 pmol/lane.

With hepatopancreas microsomal preparations from the other invertebrates studied, immunoreactivity at a similar molecular mass to that of the spiny lobster cytochrome P450 was detected with the slipper lobster (figure 2.1 and figure 2.2). This lobster is in the same infraorder as the spiny lobster. Cross-reactivity at higher molecular mass was detected with samples from the American lobster, but there was no detectable cross-reactivity with the other invertebrate samples studied (figure 2.1 and figure 2.2). Many factors effect the cytochrome P450 levels in marine invertebrate species (Stegeman and Hahn, 1994). Failure to detect immunoreactive proteins may be due not only to lower levels of overall cytochrome P450 contained in the hepatopancreas or digestive gland of the invertebrates studied, but may also be related to differential expression of a particular cytochrome P450 isoform.

Table 2.1
Classification and cytochrome P450 Contents of Hepatic Preparations of the Species Studied

Classification	cytochrome P450 content (nmol/mg protein)
Phylum Arthropoda	
Subphylum Chelicerata	
Class Xiphosura	
Limulus polyphemus, the horse shoe crab[1]	0.41
Subphylum Mandibulata	
Class Crustacea	
Order Decapoda	
Suborder Dendrobranchiata	
Infraorder Penaeidea	
Superfamily Penaeoidea	
Family Penaeidae	
Penaeus aztecus, the brown shrimp[1]	0.10
Suborder Pleocyemata	
Infraorder Palinura	
Superfamily Palinuroidea	
Family Palinuridae	
Panulirus argus, the Florida spiny lobster[2]	1.30
Family Scyllaridae	
Scyllarides nodifer, the slipper lobster[1]	0.06
Infraorder Astacidea	
Superfamily Nephropoidea	
Family Nephropidae	
Homarus americanus, the American lobster[2]	0.91
Infraorder Brachyura	
Superfamily Portunoidea	
Family Portunidae	
Callinectes sapidus, the blue crab[2]	0.33
Phylum Chordata	
Class Chondrichthyes	
Order Rajiformes	
Family Rajidae	
Raja eglanteria, the clear-nose skate[1]	0.53
Class Osteichthyes	
Order Siluriformes	
Family Ictaluridae	
Ictalurus punctatus, the channel catfish[1]	0.23
Order Atheriniformes	
Family Cyprinodontidae	
Fundulus heteroclitus, the killifish[1]	0.36
Order Perciformes	
Family Centropomidae	
Centropomus undecimalis, the snook[3]	0.18
Class Mammalia	
Order Rodentia	
Family Muridae	
Rattus rattus, control Sprague-Dawley rat[1]	1.10
phenobarbital-induced rat[1]	1.80

[1]Microsomes prepared from fresh liver or hepatopancreas. [2]Fractions prepared from fresh hepatopancreas. [3]Microsomes prepared from frozen livers.

Hepatic microsomes from the one member of the chondrichthyes class that were screened, the clear-nose skate, cross-reacted with the spiny lobster anti-cytochrome P450 antibody (figure 2.2).

Hepatic microsomes from all of the bony fish studied cross-reacted and gave signals in the 45-66 kDa region, with the strongest signals from the killifish microsomal samples followed by the catfish (figure 2.1 and figure 2.2). In other experiments with different microsomal preparations from the clear-nose skate and the snook, stronger signals were observed than those shown in figure 2.1 (figure 2.2). An antibody to rat cytochrome P450 2B1 and one to scup cytochrome P450 2B have been shown to cross-react with microsomes from the killifish, the little skate and the channel catfish (Stegeman and Hahn, 1994).

Microsomal fractions from control and phenobarbital-induced rats showed cross-reactivity to anti-cytochrome P450 2L in the 45-66 kDa range (figure 2.1 and figure 2.2). Interestingly, of the cytochrome P450s available in the data bank for comparison, cytochrome P450 2L shows the most similarity to the rat cytochrome P450 2D4.

These results suggest that cytochrome P450 in the spiny lobster hepatopancreas may share similar epitopes with cytochrome P450s in the slipper lobster, and possibly the American lobster, but that other invertebrates screened for cytochrome P450s with similar epitopes were possibly not present or were present in amounts below the limit of

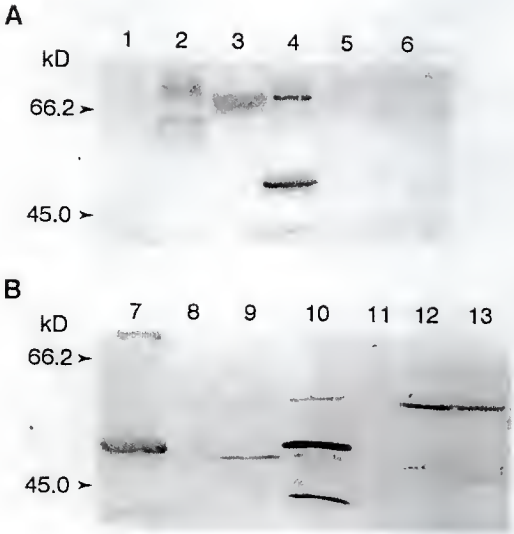


Figure 2.1. Western blots of microsomes from several species, probed with anti-spiny lobster P450. In each lane, 200 µg of protein was loaded. Lane 1, blue crab; 2, American lobster; 3, slipper lobster; 4, spiny lobster; 5, brown shrimp; 6, horse-shoe crab; 7, spiny lobster; 8, clear-nose skate; 9, catfish; 10, killifish; 11, snook; 12, control rat; 13, phenobarbital-induced rat. The migration of molecular mass markers 45 and 66.2 is shown.

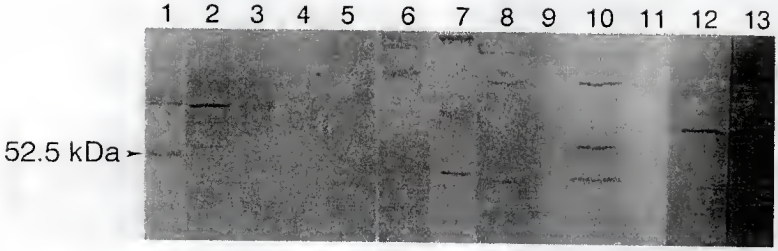


Figure 2.2. Composite picture of various Western blots done with invertebrate and vertebrate microsomal fractions. Arrow point to the spiny lobster cytochrome P450 at an apparent molecular mass of 52.5 kDa. Lane 1, female spiny lobster M1 fraction; 2, slipper lobster microsomes; 3, American lobster M1 fraction; 4, blue crab M1 fraction; 5, brown shrimp microsomes; 6, horse-shoe crab M1 fraction; 7, clear-nose skate microsomes; 8, snook liver microsomes; 9, catfish liver microsomes; 10, killifish liver microsomes; 11, empty lane; 12, control rat liver microsomes; 13, phenobarbital-induced rat liver microsomes.

detection. *In vivo* studies have shown that the American lobster and the spiny lobster metabolize benzo(a)pyrene very differently. Very slow cytochrome P450-dependent monooxygenation of benzo(a)pyrene occurs in the American lobster, but rapid monooxygenation of benzo(a)pyrene in the spiny lobster (James and Little, 1980). These differences probably reflect the cytochrome P450 composition of hepatopancreas in the two species. It would be important to isolate microsomes from other crustacea in the suborder Pleocyemata and to determine if these cross-react with the cytochrome P450 antibody to the spiny lobster form.

However, even with spiny lobster microsomes, the level of cross-reactivity may be related to the molting stage of the animal. Our laboratory has found wide variation in cytochrome P450 content in the hepatopancreas of the spiny lobster, and variations such as these may well affect attempts at quantification using Western blot techniques. As is apparent by examination of table 2.1, different amounts of cytochrome P450 were present for electroblotting. It is possible that some samples had levels of immunoreactive cytochrome P450 below the detection limit. Nevertheless, this antibody may be used to screen an expression library from the slipper lobster or other species which demonstrate cross-reactivity. Such heterologous probes are very valuable where information about the primary sequence of the target protein is unknown.

The spiny lobster antibody also cross-reacted with microsomes from cartilaginous and bony fish and from rat. Why these species would share an epitope with the spiny lobster is unknown, but may be related to the incidence of expression of the cytochrome P450 2 family. Immunlogical relationships and other molecular data relating to invertebrates will not only provide insight into the phylogenetic relationships of invertebrates, but can serve as out-groups in phylogenetic analysis of mammalian systems (Nei, 1987).

The hepatopancreas of crustaceans is composed principally of four cell types: the E (Embryonalenzellen= embryonic), R (Restzellen= absorption), B (Blasenzellen= proteases) and F (Fibrillenzellen= peroxidases; Gibson and Barker, 1979). Immunocytochemical studies of the spiny lobster hepatopancreas seem to reveal a defined distribution pattern for cytochrome P450 (figure 2.3). Immuno-reactivity appears to be localized in particular cells lining the hepatopancreas. Furthermore, the reactivity appears to be localized at the basal end of the cell. What functional significance this localization may serve *in vivo* is unknown. We can not at present identify the cell type or types in the hepatopancreas that immuno-react with the spiny lobster anti-cytochrome P450 antibody.

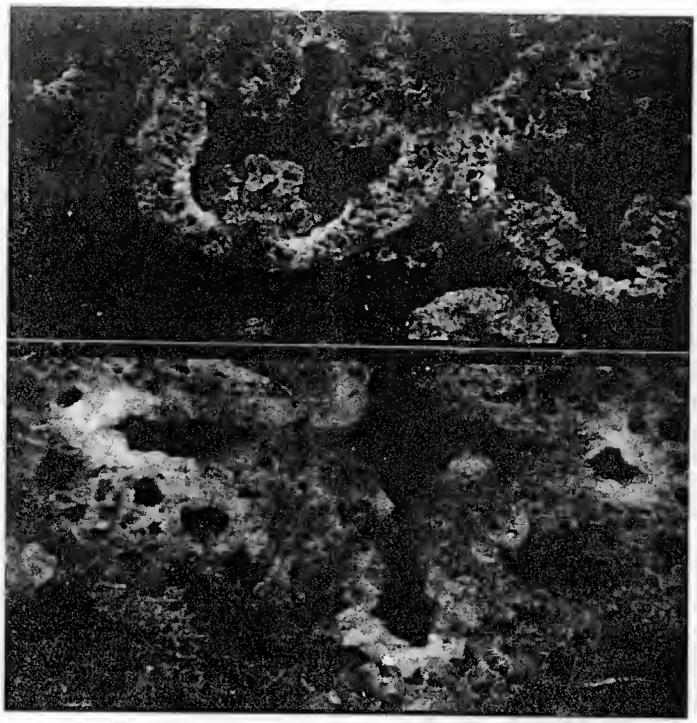


Figure 2.3. Twenty micrometer cryo-sections of spiny lobster hepatopancreas. Sections were incubated with cytochrome P450 2L antibody and stained with an FITC-linked secondary antibody. The lighter areas are cytochrome P450 in the spiny lobster hepatopancreas and seem to localize in apical cells.

CHAPTER 3
cDNA AND PROTEIN SEQUENCE OF A MAJOR FORM OF P450, CYP2L, IN THE HEPATOPANCREAS OF THE SPINY LOBSTER, *PANULIRUS ARGUS*

Introduction

Cytochrome P450s are a superfamily of important monooxygenase enzymes that are found in many animal and plant species of varying biological complexity (Nelson et al., 1993). The major function of these enzymes is to introduce oxygen into, or remove hydrogen from, an organic substrate of either endogenous or exogenous origin, usually increasing the hydrophilicity of the substrate and altering its pharmacological or physiological activity (Guengerich and Shimada, 1991). The monooxygenation of xenobiotics is usually catalyzed by members of cytochrome P450 families 1-4. The protein structure of individual members of the cytochrome P450 superfamily, as it is related to catalytic function, is an active current area of research. Considerable advances have been made in deducing the amino acid sequences and further structural details of bacterial, fungal, and some mammalian cytochrome P450s (Ortiz de Montellano, 1986; Gonzalez, 1990), but very little sequence or structural information has been published for these in nonmammalian animals (Nelson et al., 1993; Stegeman and Hahn, 1994). The few invertebrate cytochrome P450 cDNA and

deduced amino acid sequences known fall into the families 4, 6, and 10 and include the neotropical cockroach, *Blaberus discoidalis* (Bradfield et al., 1991), the fruit fly, *Drosophila melanogaster* (Nelson et al., 1993), the house fly, *Musca domestica* (Cohen et al., 1994), and the pond snail, *Lymnea stagnalis* (Nelson et al., 1993). No cytochrome P450 sequence information is available for crustacean species. Obtaining sequence information from divergent species may help to further characterize the phylogeny of this enzyme superfamily, which probably arose from the duplication of an ancestral gene (Nelson et al., 1993; Stegeman and Hahn, 1994; Nelson and Strobel, 1987; Nebert and Gonzalez, 1987; Nebert et al., 1989). Such an ancestral gene may have had a very broad substrate pool and paralogues might have evolved more specific substrate selectivites (Nelson and Strobel, 1987).

This report concerns cytochrome P450 found in the hepatopancreas, or digestive organ, of the spiny lobster, *Panulirus argus*. The spiny lobster hepatopancreas cytochrome P450 system has some interesting features (James and Little, 1984; James, 1989; James, 1990). Although microsomes isolated from the hepatopancreas contain high concentrations of spectrally measured cytochrome P450 (comparable to or somewhat higher than cytochrome P450 concentrations found in hepatic microsomes from control rats), no conclusive evidence has yet been obtained for the presence of an NADPH-cytochrome P450 reductase in spiny lobster hepatopancreas

microsomes, although low cytochrome c reductase activity is present (James, 1989). The lack of measurable NADPH-cytochrome P450 reductase may be because any cytochrome P450 reductase present undergoes proteolysis during the preparation of microsomes (James, 1990). It has not been possible to measure NADPH-cytochrome P450 reductase in spiny lobster hepatopancreas microsomes by immunological methods, as these microsomes do not contain any proteins which cross-react with an antibody to rat or rabbit NADPH-cytochrome P450 reductase (unpublished observations).

Additionally, there is no evidence that spiny lobster cytochrome P450s can be induced by treatment with polycyclic aromatic compounds, although polycyclic aromatic compounds are rapidly metabolized by the spiny lobster (James and Little, 1984; and James, unpublished observations).

In previous studies, a spiny lobster fraction (given the trivial designation D_1) was partially purified from hepatopancreas microsomes by chromatography and the catalytic activities of this cytochrome P450 with benzphetamine, ethoxycoumarin, aminopyrine, testosterone, progesterone, benzo(a)pyrene and resorufin ethers were measured in the presence of rat NADPH cytochrome P450 reductase (James, 1990). The present paper reports a 39 amino acid N-terminal sequence of the cytochrome P450 protein found in the D_1 fraction and the sequence of a CYP cDNA cloned from hepatopancreas mRNA by polymerase chain

reaction (PCR) techniques, using primers to this N-terminal sequence.

Materials and Methods

Isolation of cytochrome P450 Samples for Sequence Analysis

A partially purified cytochrome P450 D_1 fraction (11.5 nmol spectrally measured cytochrome P450/mg protein) was obtained from spiny lobster hepatopancreas microsomal fractions by ion-exchange, hydrophobic, and absorption chromatography as described previously (James, 1990). Duplicate samples of the D_1 preparation were subjected to SDS-PAGE in one dimension by the method of Laemmli (Laemmli, 1970), as shown in figure 3.1. One gel was stained with Coomassie blue and analyzed densitometrically (ISCO Model 1312) to determine the percentage of protein in each band. The major band, at molecular weight 52,500 (see figure 3.1), was examined for sequence analysis.

Proteins were then electrophoretically transferred from an unstained gel to an Immobilon PVDF (polyvinylidene fluoride) membrane (Millipore, Bedford, MA) in the Towbin buffer system (Towbin et al., 1979). Proteins were localized on the PVDF membrane by Coomassie blue staining and the membrane stored at -20°C until sequencing. N-terminal amino acid sequence analysis was carried out at the University of Florida Protein Chemistry Core facility in the

Interdisciplinary Center for Biotechnology Research (ICBR). The band of molecular mass 52,500 daltons from the PVDF membrane (about 4.5 µg protein) was applied to an Applied Biosystems Model 470A gas-phase protein sequencer with an on-line analytical HPLC system. The peptide sequence data was compared with sequences present in the Genetics Computer Group (GCG, Madison, WI) protein database, using FASTA computer programs (Dayhoff et al., 1983; Devereux et al., 1984; Pearson and Lipmann, 1988), as well as the National Center for Biotechnology Information (NCBI), using the BLAST network service.

Preparation of RNA, mRNA, and cDNA

The hepatopancreas from a male spiny lobster was removed and a 1-g sample was homogenized in a guanidine isothiocyanate-containing buffer following the methods of Chirgwin *et al.* (Chirgwin et al., 1979). Total RNA was isolated by centrifugation through a CsCl cushion. Polyadenylated RNA was fractionated using an oligo(dT) affinity push column (Stratagene Cloning Systems, La Jolla, CA). The mRNA, 5 µg, was incubated with reverse transcriptase (1000 units, AMV, Life Technologies) in the presence of 500 µm dATP, dCTP, dGTP, and dTTP (dNTP mix), 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 µg *Not* I primer/adapter (Life Technologies, Inc., Gaithersburg, MD) in a total volume of

20 µl (Okayama and Berg, 1982; Gubler and Hoffman, 1983). After incubation at 42°C for 80 min, the reaction mixture was placed on ice. A sample, 18 µl, was added to 25 mM Tris-Cl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10 mM ammonium sulfate, 0.15 mM β-NAD^+, 0.25 mM dNTP mix, 1.2 mM dithiothreitol, 10 units of *Escherichia coli* DNA ligase, 40 units *E. coli* DNA polymerase I, and 2 units *E. coli* RNAse H in a total volume of 0.15 ml. After incubation at 16°C for 2 h, 10 units of T4 DNA polymerase was added and the incubation continued for 5 min at 16°C. The resulting blunt-ended cDNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol, 25:24:1. The DNA in the aqueous phase of the extract was precipitated by the addition of one-half vol of 7.5 M ammonium acetate and 2 vol of ice-cold ethanol. The blunt-ended cDNA was ligated to a *Sal* I adapter by incubating, in a 50 µl volume, with 50 mM Tris-Cl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 5% polyethylene glycol 8000, 1 mM dithiothreitol, 10 µg *Sal* I adapter, and 5 units T4 DNA ligase for 16 h at 16°C. The cDNA in the reaction mixture was extracted and precipitated as above. The cDNA was then incubated with 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, and 1200 units/ml *Not* I endonuclease in a final volume of 0.05 ml for 2h at 37°C. The cDNA was isolated as before and size-fractionated on a Sephacryl-500 HR column. High-molecular weight cDNA was ligated into λgt22a using the Lambda Superscript System (Life Technologies, inc.).

cDNA Library Screening

Degenerate primers HT23 and HT24 were designed against the N-terminal sequence data derived from sequencing the 52,500 band of the D_1 fraction (see table 3.1).

The sequences of these primers, and other important primers used, are shown in table 3.2. Using primers HT23 and HT24 in a polymerase chain reaction (PCR)(Compton, 1990), clone II was isolated. The relationships of the different clones obtained to each other, and to the sequence of the target cytochrome P450, are shown in figure 3.2. Clone II was 117 base pairs and coded for 39 amino acids which differed only by one residue from the N-terminal sequence of the isolated cytochrome P450 in the D_1 fraction. Clone I was then generated using an exact primer, HT26, obtained from clone II and a vector primer to the 5′ end of λgt22a. Clone I contained base pairs 1 to 93 of the target cytochrome P450. Clone IV, which coded for 851 base pairs, was generated using an exact primer, HT25, obtained from clone II, and a vector primer to the 3′ end of λgt22a. Clone III, which represents a cDNA corresponding to all of the coding region of the mRNA of this cytochrome P450, was isolated using HT23 and MJ10, primers derived from exact sequence data in clone V (table 3.2). Clone VI was obtained using this primer set, but represents an incomplete clone. All coding regions of the target cytochrome P450 were represented by at least three independent clones and all

clones were sequenced at least twice. In this manner, a consensus sequence was obtained. The PCR tubes contained the following: 5 µl cDNA library in 10 mM $MgSO_4$ (2.9×10^{10} plaque-forming units/ml), 10 µl of PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.4, 15 mM $MgCl_2$, and 1 mg gelatin/ml), 1 µl of a solution containing 20 mM dNTP mix, and 100 pmol each of the degenerate primers or 30 pmol each of nondegenerate primers. The volume was made up to 99 µl with sterile, deionized water and the reaction tubes were heated at 94°C for 5 min. *Taq* DNA polymerase (5 units, Promega, Madison, WI) was then added for a final volume of 100 µl and the reaction tubes were heated and cooled for 35 cycles under the following temperature regime: 94°C for 1 min (denaturing), 51°C for 2 min (annealing), and 72°C for 3 min (elongating). A final 10-min extension period at 72°C was included.

Cloning and Sequencing of PCR Products

PCR products were cloned into pGEM-T (Promega) and used to transform competent JM109 cells. Plasmid templates were prepared for sequencing using the Wizard Mini-Prep system (Promega). Manual dideoxy sequencing was done using the Sequenase Sequencing Kit (USB, Cleveland, OH). Additional sequencing was done by the ICBR Sequencing Core located at the University of Florida.

RT-PCR Experiments

Total RNA was isolated from the spiny lobster hepatopancreas as described above. Ten micrograms of RNA were used in the following reaction: 100 pmol of an oligo dT primer to a final volume of 6 µl diethylpyrocarbonate-treated water. The mixture was heated at 65°C for 10 min and then placed on ice. To this mixture was added 2 µl of PCR buffer, 1µl of 20 mM dNTP mix, 1 µl of RNase inhibitor (Rnasin, Promega, Inc.), 1 µl of Superscript Reverse Transcriptase (200 units, Life Technologies, Inc.) and the reaction volume brought up to 20 µl with DEPC water. The reaction mixture was incubated at 42°C for 2 hours. Portions of this reaction, 1 µl, were used in the PCR reaction using primers HT23 (figure 3.2, 100 pmol) and oligo dT (100 pmol) under the reaction conditions described above, but with the annealing temperature of 45°C. A portion of the PCR product, 1 µl, was then nested using primers HT25 and MJ11 with an annealing temperature of 51°C.

Northern Blot Analysis

Ten micrograms of RNA isolated from the hepatopancreas of the spiny lobster were denatured following the methods of McMasters and Charmichael (1977). After electrophoresis in 1.1% agarose/ 10 mM sodium phosphate buffer, pH 7.0, the RNA was transferred using a vacuum blotter to a .45 µm Magna

nylon membrane (MCI, Westborough, MA) using a vacuum blotter. The membrane was probed with a ^{32}P-dCTP labeled PCR product corresponding to the first 705 base pairs of CYP2L. This probe was labeled by random prime labeling (Pharmacia oligo labeling kit). RNA in the blots was hybridized by incubating in a solution containing 0.75 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 0.005 M EDTA, 0.1 mg/ml herring sperm DNA, 0.1% SDS for 12 hrs at 68°C. The membrane was washed three times at 68°C in 0.025 M NaCl, 0.001 M $NaH_2PO_4 \cdot H_2O$, 0.1 mM EDTA and exposed with intensifying screens to X-ray film for at least 12 hrs at -80°C.

Results and Discussion

N-Terminal Sequence of Spiny Lobster cytochrome P450

One-dimensional SDS-PAGE showed that the D_1 preparation from spiny lobster hepatopancreas microsomes contained a major protein band of 52,500-Da and some minor bands (figure 3.1).

Densitometric analysis of the Coomassie blue-stained bands (not shown) showed that the 52,500-Da bands accounted for 80% of the protein in the D_1 fraction. Microsequence analysis of about 5 µg protein from the 52,500-Da band in the D_1 preparation showed that this band accounted for 75% of the total protein. This peptide was sequenced through residue 39 (table 3.1).

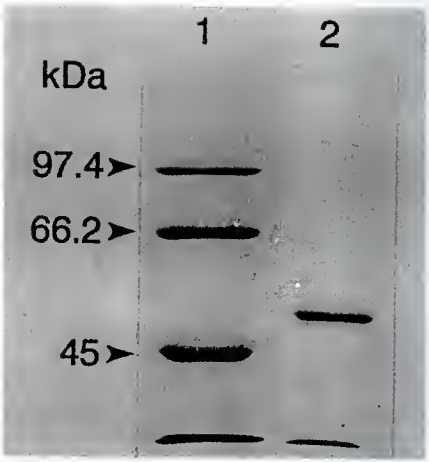


Figure 3.1. SDS-PAGE of the spiny lobster cytochrome P450-containing fraction stained with Coomassie blue. Lane 1, molecular weight standards. Lane 2, D_1 fraction, 11.5 nmol cytochrome P450/mg protein. The 52,500-Da band was shown by densitometry to contain about 80% of the total protein in this fraction.

Table 3.1.
The N-Terminal Amino Acid Sequences in a cytochrome P450-Containing fraction[a] Isolated from Spiny Lobster Hepatopancreas Microsomes

Sequence	Residues identified by microsequencing[b]
Major[c]	MLTGALLLLL VVVIVYLLDK KPSGLPPGIW GWPLVGRMP
Minor[c]	(T)WIK(K)V(L)AM

[a] The cytochrome P450-containing fraction (D_1 fraction) was isolated from spiny lobster hepatopancreas microsomes as described previously (James, 1990). The predominant protein band in this fraction, of mol. wt 52,500 on one-dimensional SDS-PAGE (see figure 1.3), was used for microsequencing.

[b] About 40 pmol was submitted for microsequencing as described under Methods. The overall repetitive yield was 94%. Parentheses indicate ambiguous amino acid assignments in the minor sequence.

[c] The major sequence shown accounted for 75% of the total protein in this band (30 pmol in the sample sequenced). The minor sequence in the 52.5 kD D_1 band accounted for 20% of the protein (8 pmol). The identity of this protein is not known.

Partial N-terminal sequence information was obtained for a minor peptide in the 52,500-Da band (table 3.1).

The first 39 amino acids obtained from N-terminal sequencing of the 52,000-Da major band in the D_1 preparation included hydrophobic amino acids characteristic of membrane-bound proteins (Black, 1992).

Comparison of this N-terminal sequence to the N-terminal sequences of other proteins in the GCG database revealed similarities to several mammalian cytochrome P450s in the 2 family (Philips et al., 1983; Labbe et al., 1988; Ueno and Gonzalez, 1990) and similarities to short stretches of the N-terminal sequences of cytochrome P450s in the 1,3, and 4 families (Kawajiri et al., 1986; Hardwick et al., 1987; Aoyama et al., 1989). From the spectrally measured cytochrome P450 content of D_1 (11.5 nmol/mg), the calculated specific content of a pure cytochrome P450 of molecular mass 52,500 Da (19 nmol/mg), and the percentage of protein in the 52,500-Da band (80%), we would expect 76% of the protein in the D_1 fraction to be cytochrome P450. This number matched well with the observed value for the major component of the D_1 preparation (75%) and provided confidence that the 39 amino acid N-terminal sequence was that of a cytochrome P450 from the spiny lobster hepatopancreas.

Table 3.2.
Sequence of some of the Primers Used to Obtain the cDNA Clones

Primer name	Sequence	Type and Location
HT23	ATG (CT)TI ACI GGI GCI (CT)TI (CT)TI (CT)T	Degenerate, a.a[1] 1-8
HT24	GGC ATI C(GT)I CCI ACI A(AG)I GGC CA	Degenerate, a.a 39-32
HT25	TTG CTG CTG GTG GTA ATA GTC TAC	Exact, a.a 9-16
HT26	TCC CCA TAT ACG TGG GGG AAG TCC	Exact, a.a 31-24
HT36	GTC AAG AAC TGG ATG GGC	Exact, a.a. 224-230
MJ10	TGT CAG GAG TGG AGT TAT	Exact, 3' to stop codon

[1] a.a, amino acid

cDNA Sequence

Degenerate primers were designed that corresponded to regions of the 39 N-terminal amino acids of the D_1 preparation. The sequences of the degenerate primers and other selected primers used are shown in table 3.2. The degenerate primers were used to PCR screen a spiny lobster cDNA library. The process was repeated with exact primers to obtain further cDNA sequences. A new exact primer was required about every 200 base pairs. Sequences obtained were melded to form a complete sequence (figure 3.2).

A separate clone which coded for all of the cytochrome P450 sequence was obtained using primers HT23 and MJ10 (see figure 3.2). This sequence has an open reading frame of 492 amino acids (calculated M_r of 56,669) and contains the heme-binding signature, residues 429 to 438, that is conserved in all CYPs (figure 3.3). The individual amino acids that are invariant in all known cytochrome P450s are highlighted in figure 3.3 with double underlining. The deduced amino acid sequence of this clone differs by 1 amino acid in the first 39 amino acids from the microsequenced D_1 peptide. Residue 11 of the clone was found to be leucine and not valine, as in the peptide. Comparison of the deduced 492-amino acid cytochrome P450 sequence with other protein sequences using the BLAST program showed that the sequence was highly

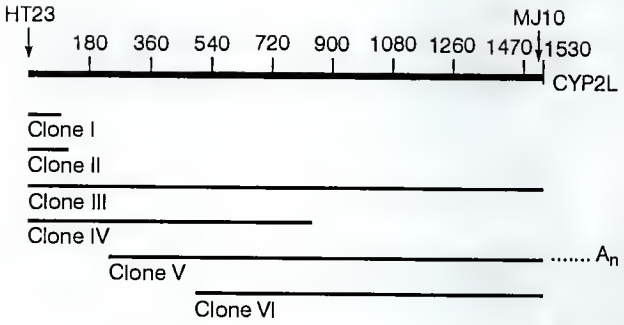


Figure 3.2. Cloning strategy showing the clones used to meld together a full-length cDNA sequence. The primers HT23 and MJ10 shown were used to generate a single clone (clone III) representing the entire coding portion of the mRNA. The sequence of these primers are shown in table 3.2.

```
ATGCTGACGGGGGCGCTGCTTTTATTGCTGCTGGTGGTAATAGTCTACCTCCTCGACAAGAAGCCATCAGGA   72
 M  L  T  G  A  L  L  L  L  L  V  V  I  V  Y  L  L  D  K  K  P  S  G    24

CTTCCCCCAGGTATATGGGGATGGCCACTGGTGGGAAGAATGCCCTCGAGGTCAAAACATCTGGCCGACCAG  144
 L  P  P  G  I  W  G  W  P  L  V  G  R  M  P  S  R  S  K  N  L  A  D  Q    48

GTTAAGCAACTGCGTAAAAAGTATGGCGACATAATCACGTGGCGCATCGGAACCAGAGTCAATGTATTCCTT  216
 V  K  Q  L  R  K  K  Y  G  D  I  I  T  W  R  I  G  T  R  V  N  V  F  L    72

TGCAACTTCAAATTGGTTAAGACGGCTCTGTCCAAGTTCGAGTGCTCAGACAGACCAGACTTCTACACTTTC  288
 C  N  F  K  L  V  K  T  A  L  S  K  F  E  C  S  D  R  P  D  F  Y  T  F    96

AAGCTTTTTGGGGAAGGCAACGATGTTGGTGTCGTCTTTAGCAATGGTGTGATGTGGCAGACGCACCGTCGC  360
 K  L  F  G  E  G  N  D  V  G  V  V  F  S  N  G  V  N  W  Q  T  N  R  R   120

TTTATTCTACGCCAGCTGAGGGACCTGGGTATGGGTAAGTCCAGACTGGAGGCCGCCATCCAGCACGAAGCC  432
 F  I  L  R  Q  L  R  D  L  G  M  G  K  S  R  L  E  A  A  I  Q  H  E  A   144

GCCTGCCTGCTGGAGGAGCTGAAGAAACACACAGACCAGCCCATGCCCCTACCAAAGTCCATAAACCTAGCT  504
 A  C  L  V  Q  E  L  K  K  N  T  D  Q  P  M  P  L  P  K  S  I  N  L  A   168

GTTCTCAACGTGATTTGGAAGCTTGTTGCAGATCACCGGTATTCGCTTCAGGATCAAGAGGGTCAATACTTC  576
 V  L  N  V  I  W  K  L  V  A  D  M  R  Y  S  L  Q  D  Q  E  G  Q  Y  F   192

ACTCAGCTTCTCACTACCACCACAGACAACATGCAGGGCTTTGCTTTGAACCTCTTCAACTACCTACCGTGC  648
 T  Q  L  L  T  T  T  T  D  N  M  Q  G  F  A  L  N  L  F  N  Y  L  P  W   216

CTTTTGATGATCACCCCAGACTTTGTCAAGAACTGGATGGGCGTTAGGGTACTACGAGACGGGGTCTGCGAG  720
 L  L  M  I  T  P  D  F  V  K  N  W  N  G  V  R  V  L  R  D  G  V  C  E   240

TTGAAAGATTACATGAAGACTTTCATCAAGGAGCACCAGGCCACGCTGGACCCGTCAAACCCAAAGGACTTA  792
 L  K  D  Y  M  K  T  F  I  K  E  N  Q  A  T  L  D  P  S  N  P  K  D  L   264

TTGGACGCCTACCTGATAGATTTGCAAGAGCGCAAGGAAGATCCTCTCTCCACAATGAACATTGAAACCGTT  864
 L  D  A  Y  L  I  D  L  Q  E  R  K  E  D  P  L  S  T  N  N  I  E  T  V   288

CGAGCCGTGATCATGGACCTGTTTGGCGCTGCTACCGAGACCACTTCAACCATGATACGATGGACGATTCTC  936
 R  A  V  I  N  D  L  F  G  A  G  T  E  T  T  S  T  M  I  R  W  T  I  L   312

TATTTGATGAAGTACCCCGAGCTGCAGGCCAAGATCCAGAGAGAGATTGATGCCGCCGTCCCTAGGGGCACC 1008
 Y  L  M  K  Y  P  E  V  Q  A  K  I  Q  R  E  I  D  A  A  V  P  R  G  T   336

TTACCCTCTCTCGAACACAAGGATAAGTTGGCGTACTTTCAGGCGACGATCCACGAGGTCCACCGCATTGTG 1080
 L  P  S  L  E  N  K  D  K  L  A  Y  F  E  A  T  I  N  E  V  N  R  I  V   360

TCTCTTGTTCCGCTTGCTGTATCCCACTACACCAACCAAGATACCGAGCTCGCCGGCTACAGACTTCCCAAG 1152
 S  L  V  P  L  G  V  S  N  Y  T  N  Q  D  T  E  L  A  G  Y  R  L  P  K   384

GGGACGCTGCTGATGAGTCATCTAGAGTGTTGCCACAGAGACCCAAGTTACTGGGAGAAGCCTAATGAGTTC 1224
 G  T  V  V  N  S  N  L  E  C  C  M  R  D  P  S  Y  W  E  K  P  N  E  F   408

TACCCGGAACATTTCCTGGACGATCAGGGCAAATTGGTCAAGAGGGAACACCTCGTCAACTTCTCTGTAGGT 1296
 Y  P  E  N  F  L  D  D  Q  G  K  F  V  K  R  E  N  L  V  N  F  S  V  G   432

CGCCGGGTATGTGTGGGCGAGTCTCTGGCCAGGATGGAGCTCTTCGTCTTCCTGTCGGCGATACTGGAGAAC 1368
 R  R  V  C  V  G  E  S  L  A  R  M  E  L  F  V  F  L  S  A  I  L  Q  N   456

TTCACCTTCTCGGCCCCTAAGGGAGAGGTGCTGCACACTGAGAAAGACCCGCAACAAATGCTATTCTCTTTT 1440
 F  T  F  S  A  P  K  G  E  V  L  M  T  E  K  D  P  Q  Q  M  L  F  S  F   480

CCCAAGCCCTATCAAGTTATCATCAGGGAGAGGGAGTGATTCCTTTGAACCAGTGGAAATGTTCATAACTCC 1512
 P  K  P  Y  Q  V  I  I  R  E  R  E  -                                   492

ACTCCTGACATTTGGCAGTATAACAAGTTGATGTCCATTCTTGTAACTTATATCTTCATTGAAGTGACTGTG 1584
ATGATA                                                                    1590
```

Figure 3.3. Nucleotide and deduced protein sequence of the spiny lobster cytochrome P450, CYP2L. The open reading frame of 492 amino acids defines a protein with a calculated molecular mass of 56,669 Da. The heme-binding signature is underlined and invariant amino acids in all known cytochrome P450s are bold and double underlined.

similar to cytochrome P450s in the 2 family but not to any non-cytochrome P450 sequences. Rat CYPs 2B1, 2B2, and 2D4 were all 36% identical at the amino acid level to the spiny lobster sequence. While several studies have shown the catalytic activity of a cytochrome P450 is not necessarily indicative of a particular cytochrome P450 family, it was of interest that previous reconstitution experiments with the D_1 cytochrome P450 showed good activity with substrates commonly monooxygenated by cytochrome P450s in the 2B family, such as testosterone (6β and 16α), progesterone (16α), benzphetamine, and aminopyrine (James, 1990; James and Shiverick, 1984).

Although the overall sequence identity of the spiny lobster cytochrome P450 with cytochrome P450s in the 2 family was less than 40%, this new form was assigned to the 2 family by the CYP nomenclature committee and given a new subfamily name, CYP2L.

Because the N-terminal sequence of the CYP2L described above was 1 amino acid different from the cytochrome P450 sequence in the D_1 fraction, obtained by microsequencing of the protein, the spiny lobster hepatopancreas cDNA library was rescreened by PCR with an exact probe to the N-terminal sequence. Other positive clones were obtained and have been partially sequenced. The deduced N-terminal amino acid sequence of one of these clones was identical to the first 39 amino acids of the D_1 cytochrome P450 protein. This

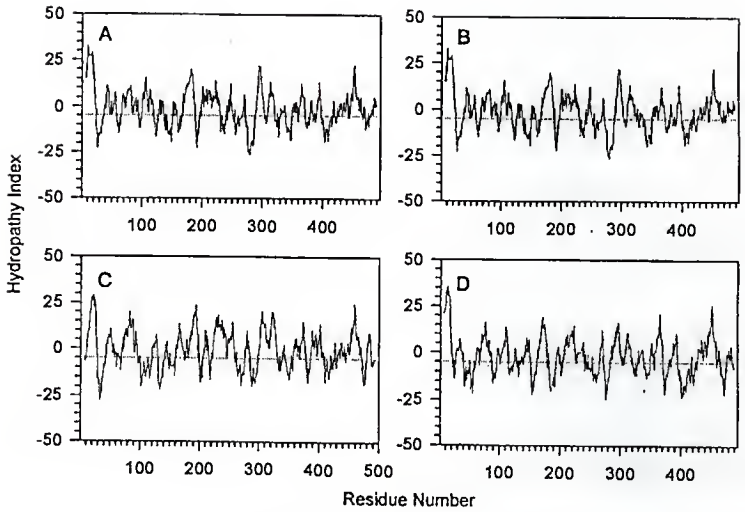


Figure 3.4. Hydropathy plots of the rat CYP2B1 (Fujii-Kuriyama et al, 1982; accession number J00719)(A), rat CYP2B2 (Mizukami et al, 1983; accession number A21162)(B), rat CYP2D4 (Matsunaga et al, 1990; accession number P13108)(c), and spiny lobster CYP2L (accession number U44826)(D). The hydropathy index computation was done using PCGENE (Intelligenetics, Mountain View, CA) with an interval (sliding window) of nine amino acids (Kyte and Doolittle, 1982).

suggests that there may be other closely related members of the CYP2L subfamily in spiny lobster hepatopancreas.

Comparison With Other 2 Family CYPs

Comparisons of hydropathy plots of CYP2L and rat CYPs 2B1, 2B2, and 2D4 indicate several structural similarities between these forms (figure 3.4). The peaks that line up in all forms appear to correspond to alpha helical regions present in 2 family members.

The alignment of primary sequences of CYP2L and rat CYPs 2B1, 2B2, and 2D4 are shown in figure. 3.5. Some regions of the sequence of spiny lobster 2L and rat 2 family CYPs show high homology, whereas other regions bear little similarity to each other. It was remarkable that the string of leucines at positions 6-11 of the spiny lobster CYP2L and the PPG cluster at spiny lobster 2L residues 26-28 were found in several members of the mammalian 2 family cytochrome P450s (Philips et al., 1983; Labbe et al., 1988; Kawajiri et al., 1986). These highly conserved portions probably contribute to the hydrophobicity of the N-terminus and its ability to associate with microsomal membranes (Black, 1992). Substrate recognition sites (SRS) have been suggested for rat 2B2 at positions 97-118 (SRS1), 199-206 (SRS2), 234-242 (SRS3), 287-305 (SRS4), 360-370 (SRS5), and 471-478 (SRS6) by Gotoh (1992). The 2L sequence has residues in common with other 2 family members in several of these substrate recognition

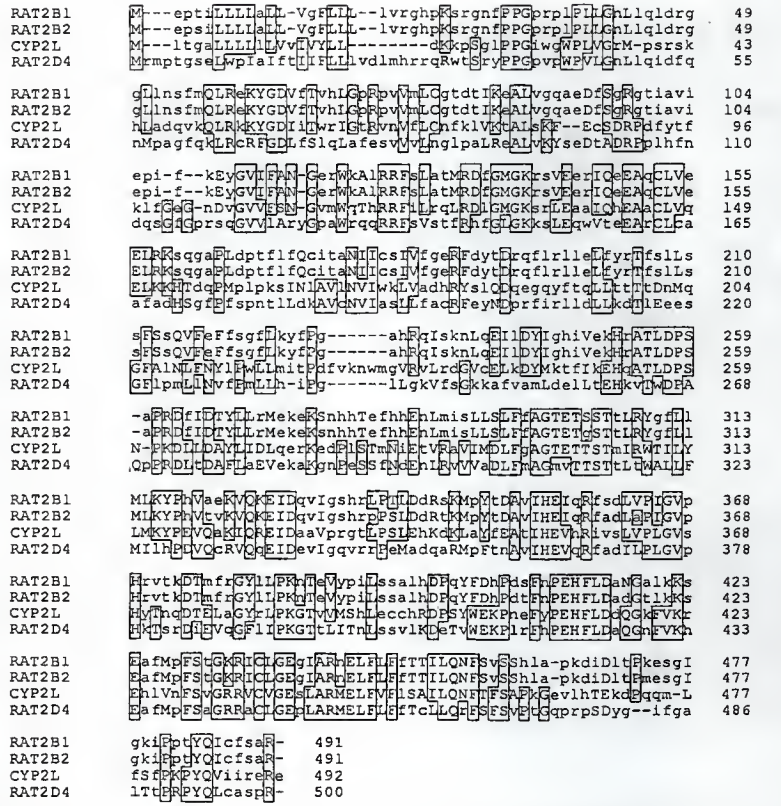


Figure 3.5. Comparison of the deduced amino acid sequence of CYP2L with that of rat CYPs 2B1, 2B2, 2B4 (accession number S19172) and 2D4. Boxes show residues that are identical between 2L and 2D4 or the 2B subfamily members, while conserved amino acids are indicated by capital letters. For the complete sequence, there were 115 amino acids (23.4%) that were identical in all five forms and 64 additional similar amino acids (13.0%). Comparing the C-terminal half (residues 250-492) of CYP2L with the C-terminal halves of 2B1, 2B2, 2B4 and 2D4, there were 73 identical residues (30.3%) and an additional 38 similar residues (15.8%). These comparisons were made using CLUSTAL V.

sites. The GV residues at CYP2L positions 106-107 fall in SRS1 and are identical in the sequences shown in figure 3.5. In SRS2, the leucine at CYP2L position 195 and the T at CYP2L position 199 are present in the rat sequences (figure 3.5). In SRS4 there were several residues found in all five sequences, i.e., LF at 295-296, AG at 298-299, T at 302, and ST at 304-305 (figure 3.5). In SRS5 residues P (364), GV (366-367) and H (369) were common in the compared sequences. It has been suggested that S at position 304 and V residues at positions 363 and 367 may contribute to substrate binding (He et al., 1994). The regions designated as SRS3 and SRS6 did not have common residues in the 2 family members that were compared in figure 3.5. Other highly conserved regions are found at residues 119-121, 252, 257-258, 325, 327-329, 408, 410-415, 438-439, 442-443, 445-447, 449, and 454-455 (figure 3.5). Several investigators have noted that regions of the C-terminus of cytochrome P450 sequences show greater homology overall than N-terminal regions (Kalb et al., 1988; Lewis, 1995). This is true for the CYP2L sequence with selected rat 2B and 2D sequences (figure 3.5).

Northern Blot and RT-PCR

Northern blot analysis reveals a primary transcript of about 1.8 kB in size (figure 3.6).

This result serves to confirm that the message for CYP2L1 is present in the spiny lobster hepatopancreas.

Furthermore, there is an indication of a second transcript around 1.5 kb (figure 3.6). Experiments using RT-PCR also suggest the existence of a second transcript (figure 3.7), however these results require confirmation.

There are numerous examples of alternatively spliced cytochrome P450 messages in the 2 family (Kimura et al., 1989; Miles et al., 1989 Yamano et al., 1989; Lacroix et al., 1990). What function such a transcript may serve is unknown. Our lab consistently notes a 30 kDa band that immunoreacts with a polyclonal antibody to CYP2L on Western Blots (Boyle and James, 1996).

The only other invertebrate cytochrome P450s that have been sequenced and assigned families to date have been from insects and a pond snail and are in the 4,6, and 10 families (Nelson et al, 1993). Thus, this is the first report of a 2 family cytochrome P450 in an invertebrate and extends the incidence of this family in the animal kingdom.

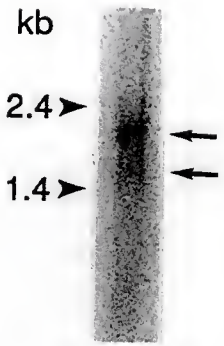


Figure 3.6. Northern blot of total RNA isolated from the hepatopancreas of the spiny lobster. Ten micrograms of total RNA were blotted onto a nylon membrane and probed with a 32P-CTP-labeled probe, as described in Methods. Arrows mark a possible 1.5 kb message (lower arrow) and about a 2.1 kb message (upper arrow).

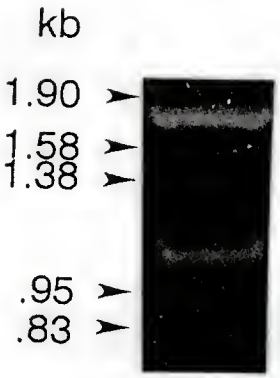


Figure 3.7. RT-PCR of total RNA isolated from the spiny lobster hepatopancreas. RNA was primed with an oligo-dT primer and reverse transcribed as described in Methods. Using HT25 and MJ11, a primer just upstream to the poly-T tail, two bands were detected. The band at around 1.8 kb (upper arrow) is the expected product for a normal transcript. The product around 1.0 kb (lower arrow) may represent an alternatively spliced message.

CHAPTER 4
CATALYTIC CHARACTERIZATION OF CYTOCHROME P450 2L1 IN BACTERIAL AND YEAST EXPRESSION SYSTEMS

Introduction

Cytochrome P450 enzymes catalyze the insertion of oxygen into both endogenous and exogenous substrates found in many animal and plant species (Nelson et al., 1993). In its dual role, cytochrome P450s function as integral parts of biosynthetic pathways, such as steroid biosynthesis, and in the initial or phase I detoxification pathways of xenobiotics.

Tissues from several crustacean species are able to metabolize various steroid hormones *in vitro* (Table 4.1). Studies have shown that invertebrates possess steroid hormones similar or identical to those found in mammalian species (Burns et al., 1984; Fairs et al., 1989). Tcholakian and Eik-Nes (1971) reported that progesterone could be metabolized to 11-deoxycorticosterone (21-hydroxyprogesterone), androstenedione and to 20-hydroxyprogesterone in the "androgenic gland" of the blue crab, reactions that can be catalyzed by cytochrome P450. Ovarian tissues from the crab, *Portunus tritubeculatus*, hydroxylate progesterone in the 17α-position (Teshima and Kanazawa, 1971). The shore crab, *Carcinus maenas*,

Table 4.1. *in vitro* Steroid Metabolism in Crustacean Species

species	organ	substrate(s)	product(s)	reference
Blue crab *Callinectes sapidus*	AG	P	21OHP, 20OHP Andro	Tcholakian and Eik-Nes, 1971
Crab *Portunus trituberculatus*	Ovaries	P	17αOHP	Teshima and Kanazawa, 1971
shore crab *Carcinus maenas*	Testes	Andro	T	Blanchet et al., 1978
	VD + AG	estrone	17βOHE	
American lobster *Homarus americanus*	Testes	P	20OHP	Burns et al., 1984
Shrimp *Penaeus monodon*	Ovaries	P	20OHP	Young et al., 1992
Florida spiny lobster *Panulirus argus*	purified protein from HP	T	16αOHT, 16OHβT 6βOHT	James and Shiverick, 1984
		P	16αOHP, 6βOHP 17αOHP, 21OHP	
	AnG	Ec	20OHEc	

HP=hepatopancreas, VD=vas deferens, AG=androgenic gland, AnG=antennal gland. P=progesterone, Andro=androstenedione, Ecdysone, 21OHP=21-hydroxyprogesterone, 20OHP=20-hydroxyprogesterone, 17αOHP=17α-hydroxyprogesterone, 17βOHE=17β-estradiol, 16αOHT=16α-hydroxytestosterone, 16βOHT=16β-hydroxytestosterone, 6βOHP=6β-hydroxyprogestererone, 20OHEc=20-hydroxyecdysone.

metabolizes androstenedione to testosterone and estrone to 17β-estradiol in vas deferens and testes tissue preparations (Blanchet et al., 1978). Lachaise and Lafont (1984) demonstrated that the shore crab could metabolize ponasterone A (25-deoxy-20-hydroxyecdysone) to 25-hydroxyecdysone. American lobster testes were shown to metabolize progesterone to 20-hydroxyprogesterone (Burns et al., 1984), and shrimp, *Penaeus monodon*, ovary was also shown to metabolize progesterone to 20-hydroxyprogesterone (Young et al., 1992).

James reported that the M1 fraction from the spiny lobster hepatopancreas could metabolize a variety of substrates (Table 4.2, James, 1990; James, 1989). Two catalytically active fractions (D1 and D2) of cytochrome P450 in the spiny lobster hepatopancreas were isolated (James, 1990). The fractions have a similar apparent molecular mass and overlapping substrate preferences for benzo-a-pyrene, benzphetamine, ethoxycoumarin, testosterone and progesterone (James, 1990; James and Shiverick, 1984). Progesterone was hydroxylated in the 16α, 6β, and 21 positions, while testosterone was hydroxylated in the 16α, 16β and 6β positions (Table 4.2, James, 1990, James and Shiverick, 1984). Hydroxylation of progesterone or testosterone at the 16 or 6 position diminishes the biological activity of these steroids. The molting hormone, ecdysone, was metabolized to 20-hydroxyecdysone in mitochondria from spiny lobster antennal gland, as well as

Table 4.2. Monooxygenase Activity of Spiny Lobster Cytochrome P450 Fractions in the Presence of NADPH and NADPH-Cytochrome P450 Reductase from Rat Liver.

P450	Nanomoles product formed/min/nmol cytochrome		
Substrate	M1	D1	D2
Benzphetamine	26.3±5.3 (8)	50±15 (4)	122±62 (4)
Aminopyrine	19.8	40	76
7-Ethoxycoumarin	0.325±0.139 (4)	0.140±0.023 (3)	0.183
Methylphenoxazone	0.0189	0.004±0.001 (3)	0.005±0.001 (3)
Ethylphenoxazone	0.062±0.051 (5)	0.007±0.002 (4)	0.005±0.002 (3)
Pentylphenoxazone	0.002	0.011±0.001 (3)	0.013±0.001 (3)
Benzylphenoxazone	0.010	0.004±0.001 (3)	0.003±0.001 (3)
Benzo(a)pyrene	1.43±0.41 (5)	1.97±0.83 (5)	1.54±0.39 (4)
Testosterone 16α-	1.3	8.65±5.81 (3)	4.1
6β-	0.6	7.31±6.16 (3)	3.4
Progesterone 16α-	4.96±0.28 (8)	43.4±9.1 (3)	21.1
6β-	1.18±0.41 (8)	0.9±0.3 (3)	6.2
21-	0.67±0.42 (8)	0.47±0.02 (3)	0.41

Note: Values shown are means ±SD (n) or individual values. This data was taken from (James, 1990). M1=solubilized microsomal fractions, D1 and D2 are chromatographic fraction of the M1 material.

in gonadal tissues and hepatopancreas mitochondria (James and Shiverick, 1984).

We have cloned a cytochrome P450, cytochrome P450 2L1, from the hepatopancreas of the spiny lobster (James et al., 1996). The first 39 amino acids deduced from the DNA sequence of cytochrome P450 2L1 are nearly identical to N-terminal amino acid sequence data obtained from the D1 fraction, differing by only one amino acid (James et al., 1996). This difference, a substitution of a leucine for a valine, is a conservative change. However, a clone was obtained in which this substitution was absent (James et al., 1996).

The following study reports upon the expression of cytochrome P450 2L1 in bacteria and yeast. Functional cytochrome P450 2L1 was obtained from yeast and its catalytic activity determined using testosterone and progesterone substrates.

Materials and Methods

Spiny Lobster and Rat Protein Preparations

Microsomes were prepared from a male spiny lobster hepatopancreas as described previously (James, 1990). The "M1" fraction was prepared by stirring the microsomal fraction in 0.5% cholic acid for 1 hr at 4°C. The mixture

was centrifuged at 110,000 x g for 90 min and the dense, red liquid fraction isolated (M1 fraction, James, 1990).

Cytochrome P450 reductase was isolated from phenobarbital-treated rats (80 mg/kg for 4 days) by the method of Yasukochi and Masters (1976). Protein concentration of the various preparations described in this paper were done using the method of Lowry et al. (1951). Spectral determination of cytochrome P450 content followed the procedure of Estabrook (1972). SDS-PAGE was done using the methods of Laemmli (1970).

Construct Preparation

Two cytochrome P450 2L1 constructs were prepared for insertion into bacterial or fungal cells. The first construct, Δ0, was designed to express the entire deduced amino acid sequence of cytochrome P450 2L1. Δ0 was generated using primers MJ24 and MJ25 (table 4.3 and figure 4.1). A λgt22a cDNA library made from spiny lobster hepatopancreas (James et al., 1996) was screened using these two primers in a polymerase chain reaction (Compton, 1990). The PCR tubes contained the following: 5 µl cDNA library in 10 mM $MgSO_4$ (2.9 X 10^{10} plaque-forming units/ml), 10 µl of PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.4, 15 mM $MgCl_2$, and 1 mg gelatin/ml), 1 µl of a solution containing 20 mM dNTP mix, and 30 pmol of each primer. The volume was made up to 99 µl with sterile, deionized water and the reaction

Table 4.3. Primer Sequences Used in this Study.

Primer Name	Sequence	Comments
BRN1	AGTCGAATTCCATATGGCTCTGTTATTA GCACTTTTTTTATTGCTG CTGGTG M A L L L A V F L L L L V	5'-EcoR I, Nde I sites
MJ25	AGTCGAATTCCATATGCTGACGGGGGCG CTGCT M L T G A L L	5'-EcoR I, Nde I sites
MJ24	GCGAATTCGTGCACTCACTCCCTCTCCCT GATGA (- E R E R I I)[1]	3'-EcoR I, Sal I sites
M13	CGCCAGGGTT TTCCCAGTCA CGAC	5'-polylinker
HT26	TCCCCATAT ACCTGGGGGAAGTCC G W I G P P L G	3'-antisense
HT36	GTC AAG AAC TGG ATG GGC V K N W M G	5'-sense
HT38	ATC TTT CAA CTC GCA GAC CCC (D K L E C V G)[1]	3'-antisense
Ph94	GACTGGTTCC AATTGACAAG C	5'-AOX1 site (5' to polylinker)
Ph93	GGATGTCAGA ATGCCATTTG C	3'-AOX1 site (3' to polylinker)

[1]Residues in parenthesis are the inverse translation products

```
                EcoR I      M   L   T   G   A   L   L
MJ25 5' AGTCGAATTCCAT ATG CTG ACG GGG GCG CTG CT 3'
                    Nde I

                EcoR I          -   E   R   E   R   I   I
MJ24 5' GCGAATTCGTCGAC TCA CTC CCT CTC CCT GAT GA 3'
                  Sal I

                EcoR I      M   A   L   L   L   A   V
BRN1 5' AGTCGAATTCCAT ATG GCT CTG TTA TTA GCA GTT
                    Nde I

                            F   L   L   L   L   V
                        TTT TTA TTG CTG CTG GTG
                                CYP 2L1
```

Figure 4.1. The oligonucleotide sequence of expression primers MJ25, MJ24, and BRN1. MJ25 and BRN1 both incorporate unique EcoR I and Nde I endonuclease restriction sites to enable ligation of the PCR product into expression vectors that have these sites within the polylinker region. MJ24 incorporates unique EcoR I and Sal I sites into a PCR product. The resulting PCR product contains 5' and 3' EcoR I sites, and a 5' Nde I site and a 3' Sal I site.

tubes were heated at 94°C for 5 min. Pmo I (5 units, Boehrinher Mannheim, Inc.), a thermostable DNA polymerase with proofreading capabilities, was then added for a final volume of 100 µl and the reaction tubes were heated and cooled for 35 cycles under the following temperature regime: 94°C for 1 min (denaturing), 60°C for 2 min (annealing), and 72°C for 3 min (elongating). A final 10-min extension period at 72°C was included. A full length clone was constructed and ligated into pGEM-T (Promega).

A second construct was prepared, Δ1, and was designed to replace the first 7 amino acids of cytochrome P450 2L1 with the amino acids MALLLAVF (the Barnes modification). Δ1 was generated using primers BRN1 and MJ24 (see table 4.3 and fig 4.1) in a PCR reaction using conditions identical to those used for Δ0. Δ1 was also ligated into pGEM-T.

Bacterial Expression Vectors

Δ0 and Δ1 were excised from pGEM-T using either Nde I and Sal I endonucleases or only EcoR I endonuclease, depending upon which bacterial expression vector was to be used (see table 4.4 for characteristics of the various expression vectors used in this study). The Nde I/Sal I endonuclease pair was used for DNA products to be directionally inserted into pET21c, pET28a or pCW. EcoR I Nde I/Sal I endonuclease reactions were as follows: 1 µg of plasmid DNA containing either the Δ0 or Δ1 construct was incubated in 50 mM Tris-

Table 4.4. Expression Vectors Used in this Study and their Attributes

Vector	Polylinker	Selection	Bacterial Strain	Tag	Promoter
pMAL-p2	EcoR I	Ampicillin	DH5α	C-MBP	tac
pCW	Nde I/Sal I	Ampicillin	DH5α	C-PH (NU)	tac
pPET21c	Nde I/Sal I	Ampicillin	BL21	C-PH (NU)	T7
pPET28a	Nde I/Sal I	Kanamycin	BL21	N-PH	T7
pPICZa	EcoR I	Zeocin	GS115	none	AOX1

C-MBP=C-terminal maltose binding protein; C-PH=C-terminal polyhistidine; NU=not used; N-PH=N-terminal polyhistidine; AOX1=Alcohol Oxidase 1.

Cl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, 1200 units/ml Nde I and Sal I endonuclease in a final volume of 0.05 ml for 2h at 37°C. The excised DNA was gel purified and portions (1/10 of the total product recovered from the gel) ligated into pCW, pET28a and pET21c vectors that had been digested and gel purified in the same manner.

EcoR I endonuclease reactions were as follows: 1 µg of plasmid DNA containing either the Δ0 or Δ1 construct was incubated in 90 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1200 units/ml EcoR I in a final volume of 0.05 ml for 2h at 37°C. The excised DNA was gel purified and a portion (1/10 of the total product recovered from the gel) was ligated into pMAL-p2.

DH5α bacterial cells were transformed with pCW and pMAL-p2 constructs, while BL21 cells were transformed with the pET vectors (table 4.4). Positive colonies were determined using PCR and either two internal primers (HT36 and MJ24 for directional inserts) or a 5′ vector primer and a internal primer (M13 and MJ24 for bi-directional inserts).

Yeast Expression Vector

Δ0 was excised from pGEM-T using EcoR I endonuclease and ligated into pPICZa, a bi-directional vector, as described above. This plasmid was then transformed into JM109 competent cells. Positive colonies were identified by PCR using a primer to the cytochrome P450 2L1 sequence

(HT38) and a primer to the vector (Ph94, Table 4.3). The PCR experiments were designed to identify the correct orientation of the DNA insert for expression. Plasmid DNA (Qiagen, Chatsworth, CA) from a positive colony was isolated and a portion of the plasmid DNA used for sequencing in order to confirm the correct orientation and sequence the cDNA .

Twenty micrograms of the plasmid DNA was digested overnight at 25°C in 20 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 50 mM K-acetate, 1 mM DTT, 200 units/ml Pme I (New England Bio Labs, Inc.) and sterile, deionized water to a final volume of 100 µl.

Constructs linearized with Pme I were used to transform GS115 cells (*Pichia pastoris*) by electroporation (Gene Pulser, BioRad, Hercules, CA). Transformed cells were grown on 2% (w/v) agar plates containing 1.0 M sorbitol, 1.0% (w/v) dextrose, 1.34% (w/v) yeast nitrogen base lacking amino acids, 4 x 10^{-5}% (w/v) biotin, 0.005% (w/v) amino acid mixture (50 mg each glutamic acid, methionine, leucine, lysine, and isoleucine per liter DI water), 0.004% (w/v). Colonies were randomly picked and grown in 3 mls of a solution (MGYH) containing 1.34% (w/v) yeast nitrogen base, 1.0% (v/v) glycerol, 4 x 10^{-5}% (w/v) biotin, 0.004% (w/v) histidine. An aliquot of the broth containing the colonies (5 µl) was removed and subjected to PCR analysis (in order to determine what colonies underwent successful integration of the cytochrome P450 2L1 construct), using an internal

primer (HT38) to cytochrome P450 2L1 and a vector primer (Ph94).

Expression of Cytochrome P450 2L1 in Bacteria

Positive colonies containing the cytochrome P450 2L1 constructs Δ0 or Δ1 were grown overnight at 37°C in 1 ml of LB (Luria-Bertani broth) containing either 1 μg ampicillin/ml LB (pCW and pMAL-p2 transformants,) or 1 μg kanamycin/ml LB (pET transformants, see table 4.4 for antibiotic requirements of the various expression vectors).

In all cases, the overnight culture was diluted 1:100 in 100 ml LB culture with the appropriate antibiotic, and the bacteria grown to a cell density of OD_{600} between 0.70 to 0.80. isopropyl thio-β-D-galactoside (IPTG) was added to the culture to a final concentration of 0.4 mM. The cultures were grown an additional 3 hrs and harvested by centrifugation (5,000 x g for 5 min, 4°C). Cell pellets were resuspended in 10 mls of buffer A (10 mM potassium phosphate, pH 7.5, 0.15 M NaCl).

Cell pellets were subjected to 20 second sonication bursts while on ice until no viscosity was evident in the solution (typically 3 to 4 bursts were required). The ruptured cells were centrifuged at 12,000 x g for 15 min at 4°C. The pellet, consisting of insoluble material or "inclusion bodies", was resuspended in 10 mls of buffer A. The supernatant, consisting of soluble proteins and cell

membrane, was centrifuged at 180,000 x g for 65 min at 4°C. The pellet from this spin was solubilized in 0.5% cholic acid for 1 hr and the mixture centrifuged at 180,000 x g for 65 min at 4°C. In addition, the inclusion body fraction was solubalized in 0.5% cholic acid, and centrifuged at 12,000 x g for 15 min at 4°C.

Cytochrome P450 2L1 expressed from the pET28a vector was purified using metal chelation chromatography. A His-Bind (Novagen) column was poured and inclusion bodies solubilized in 6 M urea were passed over the column. The pure protein was eluted in 1.0 M imidazole.

Expression of Cytochrome P450 2L1 in Yeast

A positive colony was grown in 200 mls of MGYH. After 2 days at 30°C, cells were pelleted (1,500 x g for 10 min) and brought up in 200 mls of a solution containing 1.34% (w/v) yeast nitrogen base, 1 x 10^{-5}% (w/v) biotin, 0.5% (v/v) methanol, 0.005% (w/v) histidine. Two days later (at 30°C), the cells were pelleted and resuspended in 10 mls of buffer containing .15 M KCl, 0.05 M potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.2 mM PMSF. Microsomal fractions were prepared as described previously (James, 1990) with the following modifications: after the cells were lysed in a French press, the ruptured cell solution was centrifuged at 30,000 x g to fractionate the nuclear DNA and mitochondria. The supernatant was centrifuged at 100,000 x g for 45 min at 4°C

and the microsomal pellet was resuspended in 0.25 M sucrose, 0.05 M KP_i, pH 7.4, to a final concentration of about 12 mg microsomal protein/ml buffer.

Testosterone and Progesterone Assays

Steroid metabolism studies (n=1) in both intact cells and microsomal fractions was done following the procedures of James and Shiverick (1984). Whole cells (~9.3 x 10^9, where OD_{600}= 5.0 x 10^7 cells/ml) or microsomes (.12 mg or .096 nmol/ml) were placed into a tube containing the following: 53 µM [^{14}C]-testosterone (specific activity 57 µci/µmol, Amersham, Arlington Heights) or 43 µM [^{14}C]-progesterone (specific activity 56 µci/µmol), 0.05M KP_i, pH 7.4, 5 mM $MgCl_2$, and DI water to a final volume of .25 mls.

Reactions were initiated with the addition of NADPH (2 mM, Sigma Chemical Co.) and incubated at 30°C for 20 min. Ethyl acetate (3 X 1.5 mls) was used to terminate and extract the reaction products. The ethyl acetate fractions were evaporated under N_2 and the residues brought up in 100 µl for TLC analysis.

Linear K silica (LK5DF) gel TLC plates (Whatman Int., Maidstone, England) were predeveloped in 100% MeOH to remove impurities and allowed to dry. Reaction product (50 µl) were spotted and the plates developed three times in the following system: 70:38:0.8:1.0 diethyl ether: toluene: MeOH: acetone. The plates were allowed to dry and were

subjected to autoradiography. Steroid standards purchased from Sigma Chemical CO. (St. Louis, MO) and Steraloids (Wilton, NH) were used.

Immunoquantitation of cytochrome P450 in Yeast Microsomes

Microsomal protein, 1 and 5 µg, was subjected to SDS-PAGE. The gel was electroblotted onto PVDF membrane as described previously (James et al, 1996). A primary antibody (10 µg serum/ml tris-buffered saline, 0.05% (v/v) tween-20; a 1:500 dilution) to a major form of cytochrome P450 from the spiny lobster hepatopancreas (Boyle and James, 1996) was used to detect cytochrome P450 2L1 in yeast microsomes. This antibody was incubated overnight at 4°C with wild-type microsomes (1 µg antibody to 4 µg microsomes) and centrifuged the next day for 10 minutes at 14,000 x g. The supernatant was used in the Western blot. The secondary antibody was a goat-anti-rabbit antibody (1:3000 dilution)conjugated to alkaline phosphatase (BioRad). Desitometric analysis of the Western blot and of the TLC autoradiographs was done using an electrophoretic image band analysis system (Bioimage).

Results

Bacterial and Fungal fractions

SDS-PAGE of bacterial whole cell lysates show an inducible protein product with an apparent molecular mass of approximately 50 kDa (figure 4.2). When western blot analysis of whole cell lysates is done, an immunoreactive band is seen in the 50 kDa region (figure 4.3).

Solubilization of the bacterial membranes with cholic acid produces a protein with an apparent molecular mass approximately 58.5 kDa (figure 4.2). In addition, solubilization of the inclusion bodies also liberates a protein of an apparent molecular mass approximately 58.5 kDa (figure 4.2). Metal chelation chromatography with inclusion bodies solubalized in 6 M urea produces the same results, that is, a single band at an apparent molecular mass of 58.5 kDa (figure 4.4).

An estimate of the amount of cytochrome P450 present in the yeast microsomes was obtained using a polyclonal antibody to spiny lobster cytochrome P450 2L (Figure 4.5). We estimate that the transformed yeast produce between 0.02 and 0.05 pmole of cytochrome P450 2L1/μg yeast microsomal protein.

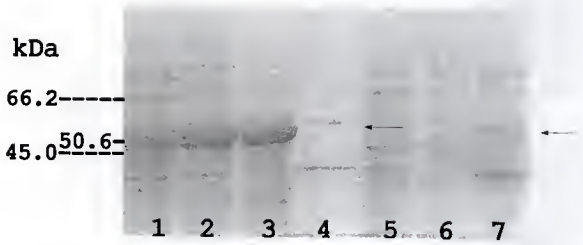


Figure 4.2. SDS-PAGE of induced bacterial cells (BL21) expressing cytochrome P450 2L1 from the expression vector pET28a. Lane 1, 500 µl of bacterial cells in SDS-PAGE running buffer; 2, 12,000 x g pellet of the culture; 3, 12,000 x g pellet solubalized in 0.5% cholic acid; 4, 12,000 x g supernatant from the lane 3 treatment; 5, 12,000 x g pellet from lane 3 treatment; 6, 180,000 x g supernatant from lane 2 supernatant; 7, 180,000 x g supernatant from lane 3 treatment. Arrows indicate a protein approximately 58.5 kDa.

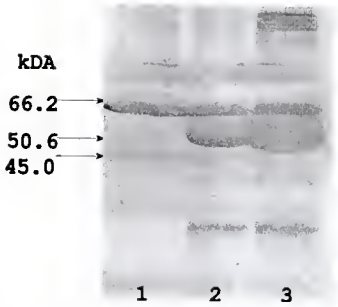


Figure 4.3. Western blot of total cell lysate from BL21 bacterial cells expressing the pET28a construct induced with 0.4 mM IPTG. Lane 1, uninduced culture; 2, culture 1 hr. 30 min. post-induction with IPTG; 3, culture 3 hr. post-induction with IPTG.

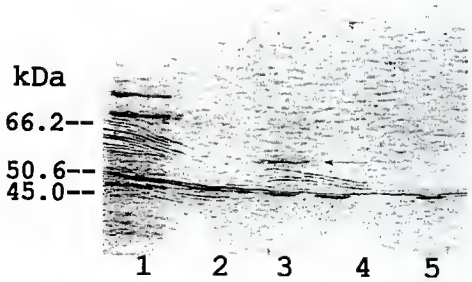


Figure 4.4. SDS-PAGE of pET28a derived cytochrome P450 2L1 expressed in bacterial cells (BL21) and purified using metal chelation chromatography. Inclusion bodies were solubalized in 6 M urea and passed over a His-bind column. The image was enhanced in order to see the pure protein (arrow), with an apparent molecular mass of 58.5 kDa. Lane 1, material that passed through the column while loading; 2, column wash; 3, first fraction following elution in a 1.0 M imidazole buffer; 4, second fraction; 5, the third fraction.

Fig. 4.5. Western blot of microsomes from yeast containing the cytochrome P450 2L1 insert. Proteins were subjected to SDS-PAGE and blotted onto a PVDF membrane as described in the Methods section. Densitometric analysis of the microsomes from yeast expressing cytochrome P450 2L1 indicate a cytochrome P450 concentration of about 0.02 pmol cytochrome P450/µg yeast microsomal protein. Control microsomes were made from wild type yeast. Purified cytochrome P450 was isolated from the hepatopancreas of the Florida spiny lobster as described previously (James, 1990). Lane 1, wild type yeast microsomes, 5 µg; 2, microsomes from yeast expressing cytochrome P450 2L1, 5 µg; 3, microsomes from yeast expressing cytochrome P450 2L1, 1 µg; 4, purified cytochrome P450, 0.35 pmol; 5, purified cytochrome P450, 0.25 pmol; 6, purified cytochrome P450, 0.15 pmol. The expressed cytochrome P450 2L1 has an apparent molecular mass of about 50 kDa.

Steroid Metabolism

[^{14}C]-testosterone was hydroxylated in the 16α position (1.37 nmol/min/nmol cytochrome P450 2L1 and 2.31 nmol/min/nmol cytochrome P450 2L1 in incubations fortified with rat cytochrome P450 reductase) by microsomes from yeast expressing cytochrome P450 2L1 (Figure 4.6). Two other polar metabolites were produced in trace amounts. A more nonpolar metabolite in reference to testosterone was produced in an NADPH-dependent, rat cytochrome P450 reductase-independent manner, but was not identified.

Intact whole yeast cells expressing cytochrome P450 2L1 incubated with [^{14}C]-testosterone, produced 16α-hydroxytestosterone, the two polar unknowns, and one nonpolar unknown. Intact whole cells did not require the addition of rat cytochrome P450 reductase nor NADPH (figure 4.7).

[^{14}C]-progesterone produced a polar metabolite (2.93 nmol/min/nmol cytochrome P450 2L1 and 3.60 nmol/min/nmol cytochrome P450 2L1 in incubations fortified with rat cytochrome P450 reductase) that co-migrated with a 16α-hydroxyprogesterone standard when incubated with microsomes from yeast expressing cytochrome P450 2L1 (Figure 4.6). One other polar metabolite was apparent, but was produced in trace amounts and we were unable to accurately quantify it

using densitometric methods. A more nonpolar metabolite in reference to progesterone was produced.

Intact whole cells expressing cytochrome P450 2L1 incubated with [^{14}C]-progesterone, produced 16α-hydroxyprogesterone, the polar unknown, and a nonpolar unknown. Intact whole cells, as with the testosterone incubations, did not require the addition of rat cytochrome P450 reductase nor NADPH (figure 4.7).

Intact whole yeast cells lacking the cytochrome P450 2L1 construct (wild type) and microsomes made from these same wild type yeast, were incubated with [^{14}C]-testosterone or [^{14}C]-progesterone in separate experiments (figure 4.6 and 4.7).

Discussion

Bacterial experiments

Both Δ0 and Δ1 were expressed successfully in bacteria with the various expression vectors used, with the exception of pMAL-p2. In all cases, large amounts of cytochrome P450 2L1 were detected either by SDS-PAGE (figure 4.2) or by immunoblotting with an antibody to spiny lobster cytochrome P450 (figure 4.3). However, no functional enzyme, as determined by cytochrome P450 difference spectra, was obtained with any of the bateria strains or expression vectors used (data not shown).

In addition, strains expressing the 50 kDa band also expressed a lower band of molecular mass 30 kDa. This 30 kDa band is consistently detected in all cytochrome P450 2L1 fractions, whether they are expressed in bacteria or yeast, or isolated from the spiny lobster hepatopancreas. It is possible that a second translation initiation start signal is present in the spiny lobster cytochrome P450 2L1 cDNA.

Figure 4.2 is a representative SDS polyacrylamide gel of bacterial contents before and after induction by IPTG. This type of expression was seen for all constructs, with higher concentrations of protein being produced with expression vectors utilizing the T7 promoter. This finding was expected, as the T7 RNA polymerase will transcribe the expression vector, and no other DNA template in this system. The vectors utilizing the tac promoter will compete for the bacterial RNA polymerase, which will transcribe all DNA templates in the bacteria.

Western blot experiments with an antibody raised against a major form of cytochrome P450 in the hepatopancreas of the spiny lobster detected a major band of molecular mass 50 kDa, which is absent in uninduced bacteria harboring the pET28a-cytochrome P450 2L1 construct (figure 4.3).

Cytochrome P450 2L1 expressed in bacteria can be solubilized in cholic acid, causing the apparent molecular mass of the 50 kDa protein to shift upward to approximately 58.5 kDa (figure 4.2). Cytochrome P450 2L1 cDNA, when

translated, should produce a protein of molecular mass equal to 56.6 kDa. Proteins expressed in the pET28a system (table 4.4) however, are expressed with a poly-histidine tag at the N-terminal end of the protein, and thus the calculated molecular mass will be slightly larger than when calculated from the deduced amino acid sequence of cytochrome P450 2L1. The calculated molecular mass of the deduced cytochrome P450 2L1 cDNA is about 56.6 kDa, and the histidine tag (MGSSHHHHHHSSGLVPRGS) will add an additional 2,044 daltons of mass, for a total mass of about 58.6 kDa.

After solubilizing inclusion bodies from bacteria expressing the pET28a-cytochrome P450 2L1 construct in 6M urea, and subjecting the solubilized material to metal chelation chromatography, a single band was detected on SDS-polyacylamide gels stained with Coomassie blue (figure 4.4). The apparent molecular mass of this band was calculated at 58.5 kDa.

The discrepancy in apparent molecular mass between the impure and purified forms of expressed cytochrome P450 2L1 in bacteria, may be due to differential migrations of the cytochrome P450s in the presence of other bacterial proteins and presumably the bacterial membrane. Membrane bound proteins such as cytochrome P450 are known to run aberrantly when subjected to SDS-PAGE.

We were unable to obtain a cytochrome P450 spectrum from bacterial whole cells regardless of the expression vector used. Using a pCW construct containing the bovine

17α-hydroxylase cytochrome P450 (CYP17, kindly provided by Dr. Ronald Estabrook and Dr. Charles Fisher) and expressed under the same conditions as the cytochrome P450 2L1 construct, we obtained cytochrome P450 spectra of about 3.4 pmol cytochrome P450 17/ml of bacterial cells (density was not determined). Thus the method of expressing cytochrome P450 in bacteria under these conditions was validated.

Yeast Experiments

We were unable to obtain spectral data of the expressed cytochrome P450 2L1 protein. However, using an antibody raised against a major cytochrome P450 fraction from the spiny lobster and pre-incubated with wild type yeast microsomes, we were able to detect a single immunoreactive band in yeast microsomes expressing cytochrome P450 2L1 that was absent in wild type yeast (figure 4.5). We estimate that the content of cytochrome P450 2L1 per μg of yeast microsomal protein to be in the range of 0.02 to 0.05 pmoles.

Microsomal fractions were made using a modified procedure from that of James (1990). The initial centrifugation at 30,000 x g may have pelleted some of the microsomal fraction. We have detected 16α-hydroxylation of testosterone in the 30,000 x g pellet (data not shown). In addition, the 100,000 x g centrifugation may have not been long enough at 45 minutes. It is possible that not all of

the microsomal fraction was pelleted during this spin. Testosterone 16α-hydroxylase activity was also detected in the 100,000 x g supernatant (data not shown). Therefore, expression levels may actually have been higher than detected.

Microsomes from yeast cells expressing cytochrome P450 2L1 were incubated with [^{14}C]-testosterone (figure 4.6). Autoradiographic data from TLC separated metabolites show a co-migrating polar compound with the 16α-hydroxytestosterone standard. The production of this metabolite appears to be NADPH-dependent. Based on densitometric analysis of the 16α product, we estimate that the maximum amount produced under these assay conditions was 2.31 nmol/min/nmol of cytochrome P450 2L1.

James and Shiverick (1984) demonstrated that 16α-(8.65±5.81 nmol/min/nmol D1 cytochrome P450 and 4.1 nmol/min/nmol D2 cytochrome P450) and 6β-hydroxylation of [^{14}C]-testosterone were catalyzed by two distinct forms of purified cytochrome P450 isolated from the spiny lobster hepatopancreas (table 4.2). We obtained slightly lower turnover numbers for 16α-hydroxylation of testosterone, and detected only trace amounts of the 6β-hydroxylated steroid.

Two other polar compounds also detected in microsomes incubated with testosterone, but their identities remain unknown. One of the unknown compounds may be 6β-hydroxytestosterone, a metabolite shown to be produced when

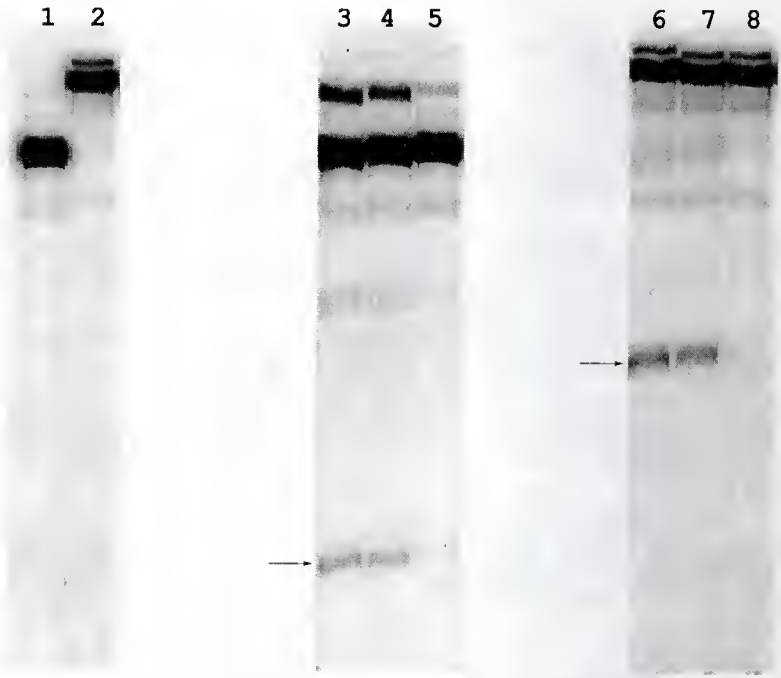


Figure 4.6. TLC separation of progesterone and testosterone metabolites produced by expressed cytochrome P450 2L1. Lane 1, testosterone incubated in control yeast microsomes in the presence of NADPH and rat cytochrome P450 reductase; 2, Progesterone incubated in control yeast microsomes in the presence of NADPH and rat cytochrome P450 reductase; 3, products of the incubation of microsomes made from *P. pastoris* expressing cytochrome P450 2L1 with [^{14}C]-testosterone in the presence of NADPH and rat cytochrome P450 reductase; 4, as for lane 3, but with no added reductase; 5, as for lane 3, but with no added NADPH or reductase; 6, products of the incubation of microsomes made from *P. pastoris* expressing cytochrome P450 2L1 with [^{14}C]-progesterone in the presence of NADPH and rat cytochrome P450 reductase; 7, as for lane 6, but with no added reductase; 8, as for lane 6, but with no added NADPH or reductase. The arrow indicates the position of 16α-hydroxy metabolites.

testosterone is incubated with the D1 fraction (James and Shiverick, 1984).

In addition, a nonpolar metabolite was also detected that migrated above testosterone in the TLC system. We believe that this metabolite is androstenedione. Androstenedione is a more nonpolar compound than is testosterone and is interconverted to testosterone. This metabolite was also detected in control incubations, and was found in higher concentrations in microsomes fortified with NADPH and rat cytochrome P450 reductase.

Incubation of [^{14}C]-testosterone with intact, whole yeast cells expressing cytochrome P450 2L1, produced the same metabolic profile as seen with the microsome incubations (figure 4.7).

When testosterone was incubated with microsomes from yeast containing the cytochrome P450 2L1 construct and TLC plates were exposed to film for one month, products co-migrating near the 6β-hydroxytestosterone standard were detected, but the signals were very weak (figure 4.6). The D1 fraction hydroxylates the 16α and 6β position of testosterone at about the same rate (Table 4.2). Cytochrome P450 2L1 expressed in yeast, however, clearly shows a preference for 16α-hydroxylation of testosterone.

NADPH-dependent 16α-hydroxylation of progesterone (3.60 nmol/min/nmole cytochrome P450 2L1) was evident in microsomes, thus indicating a cytochrome P450-dependent pathway (figure 4.6). One other polar metabolite of

progesterone was detected on the TLC plate following autoradiography but was not identified. This metabolite migrates near the 21-hydroxyprogesterone standard, and thus may be the 21-hydroxylated metabolite of progesterone. A compound more nonpolar than progesterone was detected, but was not identified.

Cytochrome P450 2L1 expressed in *Pichia pastoris* hydroxylates testosterone and progesterone predominately in the 16α position. The D1 fraction has about 1/50 the hydroxylation activity for progesterone at the 6β position relative to the 16α position (Table 4.2). Experiments using more microsomal protein from yeast containing the cytochrome P450 2L1 construct are required to detect the 6β product of progesterone.

Exogenously added reductase was not required in either the whole cell and microsomal testosterone or progesterone incubations (figure 4.6). Trant also noted that added reductase was not required in viable *Pichia pastoris* cells (Trant, 1996). When either testosterone or progesterone incubations were fortified with rat cytochrome P450 reductase, turnover numbers increased (from 1.37 to 2.31 for testosterone and 2.93 to 3.60 for progesterone). This increase in turnover number after the addition of exogenous cytochrome P450 reductase would imply that the yeast cytochrome P450 reductase is limiting in these incubations.

Incubations were also done with wild type yeast lacking the cytochrome P450 2L1 construct. Wildtype intact yeast

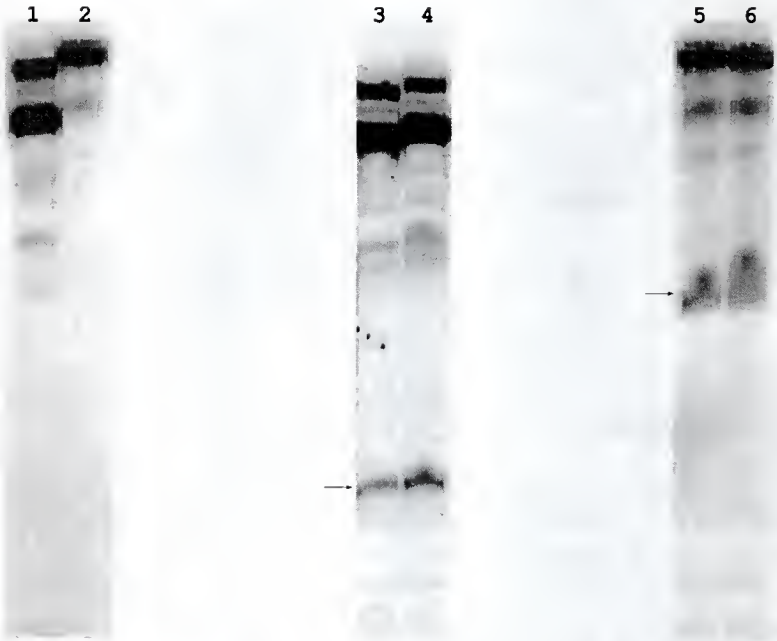


Figure 4.7. TLC separation of progesterone and testosterone metabolites produced by expressed cytochrome P450 2L1. Lane 1, testosterone incubated in control yeast whole cells in the presence of NADPH and rat cytochrome P450 reductase; 2, Progesterone incubated in control yeast whole cells in the presence of NADPH and rat cytochrome P450 reductase; 3, products of the incubation of whole cell *P. pastoris* expressing cytochrome P450 2L1 with [^{14}C]-testosterone in the presence of NADPH and rat cytochrome P450 reductase; 4, as for lane 3, but with no added NADPH or reductase; 5, products of the incubation of whole cell *P. pastoris* expressing cytochrome P450 2L1 with [^{14}C]-progesterone in the presence of NADPH and rat cytochrome P450 reductase; 6, as for lane 5, but with no added NADPH or reductase. The arrow indicates the position of 16α-hydroxy metabolites.

cells and wild type yeast microsomes failed to produce any 16α steroid metabolites when incubated with either [^{14}C]-testosterone or [^{14}C]-progesterone. These incubations were fortified with NADPH and rat cytochrome P450 reductase (figures 4.6 and 4.7).

As yet, cytochrome P450 2L1 can not be clearly associated with the D1 or D2 fractions. Further experiments are required using other substrates metabolized by the D1 and D2 fractions to establish which isoform, the D1 or D2, is represented by the translated product of the Cytochrome P450 2L1 construct in yeast. We have recently sequenced an additional cDNA form of cytochrome P450 cloned from a cDNA library made from the spiny lobster hepatopancreas, cytochrome P450 2L2, and there is evidence for two additional sequences (unpublished data). It is possible that cytochrome P450 2L2 or one of the other 2 partial clones are represented by the D1 or D2 fractions.

Heterologous expression of cytochrome P450s from the spiny lobster in *Pichia pastoris* will perhaps enable the correlation of catalytically active fractions from microsomes to individual isoforms cloned from the spiny lobster and expressed in the yeast.

CHAPTER 5
SUMMARY OF RESULTS

In the preceding chapters I have presented data regarding the cytochrome P450 monooxygenase system in the Florida spiny lobster, *Panulirus argus*. The spiny lobster is a unique animal system in which one might address a variety of biological questions. *Panulirus* is especially suited for the study of cytochrome P450 enzymes because there is a high concentration of the enzyme in the hepatopancreas, the animal's digestive organ. In addition to these high levels of P450, the spiny lobster is a species that is consumed by humans, and unlike many other animal models, data obtained from the study of this animal has the added benefit that data obtained from studies of trophic transfere may give some indication of how xenobiotics are transferred to humans that consume the spiny lobster. Finally, like all known crustacean species, the spiny lobster does not undergo the process of carcinogenesis. Studying basic mechanisms of chemical carcinogenesis in such a species may lay the groundwork for a better understanding of this phenomenon.

In chapter two, data were presented showing the cross-reactivity of microsomes from a variety of species to a polyclonal antibody generated against the major form of

polyclonal antibody generated against the major form of cytochrome P450 in the spiny lobster. Although the slipper lobster was the only invertebrate demonstrating cross-reactivity, failure of the others to cross-react may be due to the very low levels of cytochrome P450 in these microsomes or perhaps due to the absense of similar cytochrome P450s in these species. Crustacean species have a very wide range of cytochrome P450 concentrations. The spiny lobster and the crayfish exhibit very high levels (about 1.0 nmol P450/mg microsomal protein) to very low levels, as found in the chiton (around 0.1 nmol P450/mg microsomal protein). In addition, seasonal variation, as well as the sex of the animal and its nutritional status often effect P450 levels.

Most fish microsomes cross-reacted with the spiny lobster P450 antibody, in particular, the killifish microsomes. These results indicate that spiny lobster cytochrome P450 may share epitopes with the fish P450s. It has been proposed that the 2 family arose in response to animal-plant warfare when animals began terrestrial colonization. However, with a trout CYP2K form reported (Nelson et al, 1993) and now a spiny lobster CYP2L form, perhaps the 2 family has existed longer than previously believed. Or it is possible that the fish and invertebrate 2 forms represent cytochrome P450 descendants from an

ancestral gene that gave rise to the current 2 family members.

As pointed out in chapter 3, CYP2L is most similar to rat CYPs in the 2B and 2D families. It is possible that this similarity to both families is due to CYP2L being more similar to an ancestral gene that gave rise to the 2B and 2D families. Other features of the CYP2L form reveal the presence of a highly conserved heme-binding domain and several amino acids that are invariant in all known cytochrome P450 enzymes. A second clone has recently been sequenced and has been assigned the name CYP2L2. CYP2L1 and CYP2L2 are about 55% identical. CYP2L1 was modified for expression in yeast.

Chapter 4 presents data pertaining to expression of CYP2L1 in the methylotrophic yeast *Pichia pastoris*. Yeast were transfected with a linearized vector containing a 5' AOX promoter preceding the CYP2L1 code and integrated into the yeast genome. After induction of the P450 gene using the AOX promoter, cells were collected and microsomal fractions made. These fractions, as well as living cells, were used in incubations with radiolabeled testosterone and progesterone. Microsomal 16α-hydroxylation of both substrates was shown to be NADPH-dependent and only the 16α product was detected. Living yeast did not require the addition of NADPH and only yielded the 16α products. Neither microsomes nor

in tact yeast required the addition of cytochrome P450 reductase, indicating the presence of endogenous reductase that was functional with the expressed spiny lobster cytochrome P450. Studies using purified testosterone gave some indication of 16β and 6β production.

Other cytochrome P450s have been detected in the hepatopancreas of the spiny lobster and it would be interesting to express these different forms in yeast as well. It has been shown that the spiny lobster "M1" fraction can metabolize a wide range of substrates. What P450 forms are responsible for these reactions are not yet defined, and an expression system like *Pichia* offers an excellent expression system to make such determinations.

REFERENCES

Anundi, L., Lahteenmaki, T., Rundgren, M., Moldeus, P., and Lindros, K. O. (1993) Zonation of acetominophen metabolism and cytochrome P450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. Biochem. Pharmacol. 45:1251-1259

Aoyama, T., Yamano, S., Waxman, D. J., Lapenson, D. P., Meyer, U. A., Fischer, V., Tyndale, R., Inada, T., Kalow, W., Gelboin, J. V., and Gonzalez, F. J. (1989). Cytochrome P-450 hPCN2, a novel cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver. J. Biol. Chem. 264:10388-10395

Argos, P. (1987) A sensitive procedure to compare amino acids sequences. J. Mol. Biol. 193(2):385-396

Baum, R.M. (1985) New variety of street drugs poses growing problem. C & EN Sept 9, pp. 7-16

Black, S.D. (1992) Membrane topology of the mammalian P450 cytochromes. FASEB J. 6:680-685

Blanchet, M. F., Ozon, R., and Meusy, J. J. (1972) Metabolism of steroids, *in vitro*, in the male crab *Carcinus maenas*. Comp. Biochem. Physiol. 41B:251-261

Boyle, S. M. and James, M. O. (1996) Cross-reactivity of an antibody to spiny lobster P450 2L with microsomes from other species. Mar. Environ. Res. 42:1-6

Bradfield, J.Y., Lee, Y-H., and Keeley, L.L. (1991) Cytochrome P450 family 4 in a cockroach: Molecular cloning and regulation by hypertrehalosemic hormone. Proc. Natl. Acad. Sci. USA 88:4558-4562

Brodie, A.M. (1993) Aromatase, its inhibitors and their use in breast cancer treatment. Pharmacol-Ther. 60(3):501-15

Brosen, K. (1993) The pharmacogenetics of the selective serotonin reuptake inhibitors. Clinical Invest. 71(12):1002-9

Brown, C.A. and Black, S.D. (1989) Membrane topology of mammalian cytochrome P-450 from liver endoplasmic reticulum. J. Biol. Chem. 264(8):4442-4449

Buhler, R., Lindros, K. O., Nordling, A., Johansson, I, Ingelman-Sundberg, M. (1992) Zonation of cytochrome P450 isozymes expression and induction in rat liver. Eur. J. Biochem. 204:407-412

Burns, B. G., Sangalang, G. B., Freeman, H. C., and McMenemy, M. (1984) Bioconversion of steroids by the testes of the American lobster, *Homarus americanus*. Gen. Comp. Endocrinol. 54:422-428

Carr, W.E.S, Trapido-Rosenthal, H.G. and Gleeson, R.A. (1990) The role of degredative enzymes in chemosencory processes. Chem. Senses 15(2):181-190

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299

Cohen, M.B., Koener, J.F., and Feyereisen, R. (1994) Structure and chromosomal localization of CYP6A1, a cytochrome P450-encoding gene from the house fly. Gene 146:267-272

Compton, T. (1990) "Degenerate primers for DNA amplification" in PCR Protocols (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds.), pp. 39-45, Academic Press, San Diego, CA.

Coon, M.J., Ding, X.X., Pernecky, S.J. and Vaz, A.D. (1992) Cytochrome P450: Progress and predictions. FASEB-J. 6(2):669-73

Dahl, A.R. and Hadley, W.M. (1991) Nasal cavity enzymes involved in xenobiotic metabolism: Effect on the toxicity of inhalants. Crit. Rev. Tox. 21(5):345-372

Dayhoff, M. O., Barker, W. C., and Hunt, L. T. (1983) Establishing homologies in protein sequences. Methods Enzymol. 91:524 545

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395

den Besten, P. J., Lemaire, P., Livingstone, D. R., Woodin, B. R., Stegeman, J. J., Herwig, H. J., and Seinen, W. (1993). Time-course and dose-response of the apparent induction of the cytochrome P450 monooxygenase system of pyloric caeca microsomes of the females sea star Asterias rubens L. by benzo[a]pyrene and polychlorinated biphenyls. Aquatic Toxicol. 26:23-40

Denhardt, D.T. (1966) A membrane-filter technique for detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641-646

Escalante. B., Omata, K., Sessa, W., Lee, S.G., Falck, J.R. and Schartzman, M.L. (1993) 20-hydroxyeicosatetraenoic acid is an endothelium-dependent vasoconstrictor in rabbit arteries. Eur. J. Pharmacol. 235(1):1-7

Estabrook, R.W., Peterson, J., Barron, J. and Hildebrant, A.G. (1972) The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. Methods Pharmacol. 2:303-350

Fairs, N. J., Evershed, R. P., Quinlan, P. T., and Goad, L. J. (1989) Detection of conjugated and unconjugated steroids in the ovary, eggs, and haemolymph of the decapod crustacean *Nephrops novegicus*. Gen. Comp. Endocrinol. 4:199-208

Fujii-Kuriyama, Y. (1993) "Drug-inducible P-450s" in Cytochrome P450. Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y. eds. VCH Publishers Inc., New York. pg. 224-229

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., and Muramatsu, M. (1982). Primary structure of a cytochrome P450: Coding nucleotide sequence of phenobarbital-inducible cytochrome P450 cDNA from rat liver. Proc. Natl. Acad. Sci. USA 79:2793-2797

Gibson, G.G. and Skett, P. (1986) Introduction to Drug Metabolism, Chapman and Hall, London

Gibson, R. and Barker, P.L. (1979) The decapod hepatopancreas. Oceanogr. Mar. Biol. Ann. Rev. 17:285-346

Gonzalez, F.J. (1988) The molecular biology of cytochrome P450s. Pharmacol. Reviews 40(4):243-288

Gonzalez, F. J. (1990) Molecular genetics of the P-450 superfamily. Pharmacol. Ther. 45:1-38

Gonzalez, F. J. (1992) Control of constituatively-expressed developmentally-activated rat hepatic cytochrome P450 genes. Keio J. Med. 42:68-75

Gotoh, O. (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analysis of amino acid and coding nucleotide sequences. J. Biol. Chem. 267:83-90

Gotoh, O. (1993) "P450 genes and their expression" in Cytochrome P450. Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y. eds. VCH Publishers Inc., New York. pg. 207-223

Gubler, U., and Hoffman, B. J. (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263-269

Guengerich, F.P. and McDonald, T.L. (1990) Mechanisms of cytochrome P450 catalysis. FASEB J. 4:2453-2458

Guengerich, F.P. and Shimada. T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. Chem. Res. Tox. 4(4):391-407

Hardwick, J. P., Song, B-J., Huberman, E., and Gonzalez, F. J. (1987) Isolation, complementary DNA sequence and regulation of rat hepatic lauric acid ω-hydroxylase (cytochrome P-450LAω). J. Biol. Chem. 262:801-810

He, U., Luo, Z., Klekotka, P.A., Burnett, V.L. and Halpert, J.R. (1994) Structural determinants of cytochrome P450 2B1 specificity: Evidence for five substrate recognition sites. Biochemistry 33:4419-4424

James, M.O. (1989) Cytochrome P450 monooxygenases in crustaceans. Xenobiotica 19(10):1063-1076

James, M.O. (1990) Isolation of cytochrome P450 from hepatopancreas microsomes of the spiny lobster. Panulirus argus, and determination of catalytic activity with NADPH cytochrome P450 reductase from vertebrate liver. Arch. Bioch. Biophys. 282:8-17

James, M.O., Altman, A.H., Li, C-L. L. and Boyle S.M. (1992) Dose- and time-dependent formation of benzo(a)pyrene metabolite DNA adducts in the spiny lobster, Panulirus argus. Marine Environ. Res. 34:299-302

James, M.O., Altman, A. H., Li, C-L. J., and Schell, J. D., Jr (1995) Biotransformation, hepatopancreas DNA binding and pharmacokinetics of benzo[a]pyrene after oral and parenteral administration to the American lobster, *Homarus americanus*. Chem.-Biol. Interactions 95:141-160

James, M.O., Boyle, S.M., Trapido-Rosenthal, H.G., Carr, W.E.S., and Shiverick, K.T. (1993) Identification of a cytochrome P450 sequence in cDNA from the hepatopancreas of the Florida spiny lobster. Federation of American Societies for Experimental Biology Journal 7, A1201.

James, M.O., Boyle, S.M., Trapido-Rosenthal, Smith, W. Clay, Greenberg, R. M., and Shiverick, K.T. (1996) CDNA and protein sequence of a major form of P450, CYP2L, in the hepatopancreas of the spiny lobster, *Panulirus argus*. Arch. Biochem. Biophys. 329: 31-38

James, M.O. and Kleinow, K.M (1994) "Trophic Transfer of Chemicals in the Aquatic Environment" , in Aquatic Toxicology: Molecular, Biochemical and Cellular Perspectives. Malins, D.C. and Ostrander, G.K., eds. Lewis Publishers, Boca Raton, Florida. pg. 1-35

James, M.O. and Little, P.J. (1980) "Characterization of cytochrome P-450 dependent mixed-function oxidation in the spiny lobster, Panulirus argus." in Biochemistry, Biophysics and Regulation of Cytochrome P-450. Elsevier/North-Holland Biomedical Press.

James, M.O. and Little, P.J. (1984) Lack of effect of 3-methylcholanthrene on *in vivo* disposition and *in vitro* metabolism of benzo(a)pyrene in the spiny lobster, *Panulirus argus*. Comp. Biochem. Physiol. 78C:241-245

James, M.O., Schell,J.D., Boyle, S.M., Altman, A.H. and Cromer, E.A. (1991) Southern flounder hepatic and intestinal metabolism and DNA binding of benzo(a)pyrene (BaP) metabolites following dietary administration of low doses of BaP, BaP-7,8-dihydrodiol or a BaP metabolite mixture. Chem.-Biol. Interactions 79:305-321

James, M.O., Schell, J.D. and Magee, V. (1989) Bioavailabilty, biotransformation and elimination of benzo(a)pyrene and benzo(a)pyrene-7,8-dihydrodiol in the lobster Homarus amercanus. Bull. M.D.B.L. pp. 119-121

James, M. O., and Shiverick, K. T. (1984) Cytochrome P-450 dependent oxidation of progesterone, testosterone and ecdysone in the spiny lobster, *Panulirus argus*. Arch. Biochem. Biophys. 233:1-9

Kalb, V. F., and Loper, J. C. (1988) Proteins from eight eukaryotic P450 families share a segmented region of sequence similarity. Proc. Natl. Acad. Sci. USA 85:7221-7225

Kawajiri, K., Wantanabe, J., Gotoh, O., Tagashira, Y., Sogawa, K., and Fujii-Kuriyama, Y. (1986) Structure and drug inducibility of the human cytochrome P-450c gene. Eur. J. Biochem. 159:219-225

Kimura, H., Sogawa, K., Sakai, Y. and Fujii-Kuriyama, Y. (1989) Alternative splicing mechanism in a cytochrome P-450 (P-450PB-1) gene generates the two mRNAs coding for proteins of different functions. J. Biol. Chem. 264(4):2238-2242

Koymans, L., Donne-OP Den Kelder, G.M., Te Kopele, J.M. and Vermeulen, N.P.E. (1993) Generalized cytochrome P450-mediated oxidation and oxygenation reactions in aromatic substrates with activated N-H, O-H, C-H or S-H substituents. Xenobiotica 23(6):633-648

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132

Labbe, D., Jean, A. and Anderson, A. (1988) A constitutive member of the rat cytochrome P450IIB subfamily: Full-length coding sequence of the P450IIB3 cDNA. DNA 7:253-260

LaChaise, F., and Lafont, R. (1984) Ecdysteroid metabolism in a crab, *Carcinus maenas* L. Steroids 43(3):243-259

LaCroix, D., Desrochers, M., Lambert, M. and Anderson, A. (1990) Alternative splicing of mRNA encoding rat liver cytochrome P450e (P450IIB2). Gene 86:201-207

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-689

Lewis, D. F. V. (1995) Three-dimensional models of human and other mammalian microsomal P450s constructed from a alignment with P450102 ($P450_{bm3}$). Xenobiotica 25:333-366

Li, C-L and James, M.O. (1993) Glucose and sulfate conjugations of phenol, β-napthol and 3-hydroxybenzo(a)pyrene by the American lobster (Homarus americanus). Agat. Tox. 26:57-72

Little, P.J., James, M.O., Pritchard, J.B. and Bend, J.R. (1985) Temperature dependent disposition of ^{14}C-benzo(a)pyrene in the spiny lobster, Panulirus argus. Toxicol. Appl. Pharmacol. 77:325-333

Livingstone, D.R., Kirchin, M.A. and Wiseman, A. (1989) Cytochrome P450 and oxidative metabolism in molluscs. Xenobiotica 19:1041-1062

Lohmann, K.J., Pentcheff, N.D., Nevitt, G.A., Stetten, G.D, Zimmer-Faust, R.K., Jarrard, H.E. and Boles, L.C. (1995) Magnetic orientation of spiny lobsters in the ocean: Experiments with undersea coil systems. J. Exp. Biol. 198:2041-2048

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R. (1951) Protein measurement with the folin reagent. J. Biol. Chem. 193:265-275

Ma, Y.H., Gebremedhin, D., Schwartzman, M.L., Falck, J.R., Clark, J.E., Masters, B.S., Harder, D.R. and Roman, R.J. (1993) 20-hydroxyeicosatetraenoic acid is an endogenous vasoconstrictor of canine renal arcuate arteries. Circ.-Res. 72(1):126-136

Matsunaga, E., Ueno, M., and Gonzalez, F. J. (1990) The rat P450 IID subfamily: Complete sequences of four closely linked genes and evidence that gene conversions maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. J. Mol. Evol. 30:155-169

McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA 74:4835-4838

Miles, J.S., McLaren, A.W. and Wolf, C.R. (1989) Alternative spicing in the human cytochrome P450IIB6 gene generates a high level of aberrant messages. Nucleic Acids Res. 17(20):8241-8254

Mix, M.C. (1986) Cancerous diseases in aquatic animals and their association with environmental pollutants: a critical review. Mar. Environ. Res. 20: 1-141

Mizukami, Y., Sogawa, K., Suwa, Y., Muramatsu, M., and Fujii-Kuriyama, Y. (1983) Gene structure of a phenobarbital-inducible cytochrome P-450 in rat liver. Proc. Natl. Acad. Sci. USA 80:3958-3962

Nebert, D.W. and Gonzalez, F. J. (1987) P450 genes: Structure, evolution and regulation. Annu. Rev. Biochem. 56:945-993

Nerbert, D.W., Nelson, D.R., and Feyereisin, R. (1989) Evolution of cytochrome P450 genes. Xenobiotica 19:1149-1160

Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press.

Nelson, D.R. and Strobel, J.W. (1987) Evolution of cytochrome P450 proteins. Mol. Biol. Evol. 4:522-593

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) The P450 Superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA and Cell Biol. 12:1-51

Nhamburo, P.T., Kimura, S., McBride, W., Kozak, C.A., Gelboin, H.V. and Gonzalez, F.J. (1990) The human CYP2F gene subfamily: Identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression and chromosome mapping. Biochemistry 29:5491-5499

Oinonen, T., Mode, A., Lobie, P. E. and Lindros, K. O. (1996) Zonation of cytochrome P450 enzymes expression in rat liver: Isozyme-specific regulation by pituitary dependent hormones. Biochem. Pharmacol. 51:1379-1387

Oinonem, T., Saarikoski, S., Husgafvel-Pursianen, K., Hirvonen, A., and Lindros, K.O. (1994). Pretranslational induction of cytochrome P4501A enzymes by beta-napthaflavone and 3-methylcholanthrene occurs in different liver zones. Biochem. Pharmacol. 48:2189-2197

Okayama, H., and Berg, P. (1982) High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2:161-170

Omura, T. (1993) "Introduction: History of Cytochrome P-450" in Cytochrome P450. Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y. eds. VCH Publishers Inc., New York. pg. 1-15

Omura, T. and Sato, R. (1964) The carbon monoxide pigment of liver microsomes. J. Biol. Chem. 239:2379-2385

Ortiz de Montellano, P. R. (ed.) (1986). Cytochrome P450: Structure, Mechanism, and Biochemistry. Plenum Press, New York.

Pearson, W. R. and Lipmann, D. J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448

Pereira, B., Wu, K.K. and Wang, L.H. (1994) Molecular cloning and characterization of bovine prostacyclin synthase. Biochem. Biophys. Res. Commun. 203:59-66

Phillips, I. R., Shephard, E. A., Ashworth, A., and Rabin, B. R. (1983) Cloning and sequence analysis of a rat liver cDNA coding for a phenobarbital-inducible microheterogeneous cytochrome P450 variant: regulation of its messenger level by xenobiotics. Gene 26:41-52

Ruckpaul, K. and Rein, H. (eds.) (1984) Cytochrome P450: Structure and Functional Relationships, Biochemical and Physiochemical Aspects of Mixed Function Oxidases. Akademie, Berlin.

Saitoh, T., Xia, Y., Chen, X., Masliah, E., Galasko, D., Shults, C., Thal, L. J., Hansen, L. A., and Katzman, R. (1995) The CYP2D6B mutant allele is overrepresented in

the Lewy body variant of Alzheimer's disease. Ann. Neurol. 37:110-112

Sanghera, M.K., Simpson, E.R., McPhaul, M.J., Kozlowski, G., Conley, A.J. and Lephart, E.D. (1991) Immunocytochemical distribution of Aromatase cytochrome P450 in the rat brain using peptide-generated polyclonal antibodies. Endocrinology 129:2834-2844

Schell, J.D. and James, M.O. (1989) Glucose and sulfate conjugation of phenolic compounds by the spiny lobster (Panulirus argus). J. Biochem. Tox. 4(2):133-138

Schlenk, D., and Buhler, D. R. (1989). Determination of multiple forms of cytochrome P-450 from the digestive gland of Cryptochiton stelleri. Biochem. Biophys. Res. Commun. 163:476-480

Schwartzman, M.L., Davis, K.L., Nishimura, M., Abraham, N.G. and Murphy, R.C. (1990) The cytochrome P450 metabolic pathway of arachadonic acid in the cornea. Adv. Prostaglandin, Thromboxane and Leukotriene Res. 21:185-192

Smith, G.C.M., Tew, D.G. and Wolf, C.F. (1994) Dissection of NADPH-cytochrome P450 oxidoreductase into distinct functional domains. Proc. Natl. Acad. Sci. USA 91:8710-8714

Stegeman, J.J. and Hahn, M.E. (1994) "Biochemistry and Molecular Biology of Monooxygenases: Current Perspectives on Forms, Functions and Regulation of Cytochrome P450 in Aquatic Species" in Aquatic Toxicology: Molecular, Biochemical and Cellular Perspectives. Malins, D.C. and Ostrander, G.K., eds. Lewis Publishers, Boca Raton, Florida. pg. 87-206

Stoltz, R.A., Conners, M.S., Dunn, M.W. and Schwartzman, M.L. (1994) Effect of metabolic inhibitors on arachidonic acid metabolism in the corneal epithelium: evidence for cytochrome P450-mediated reactions. J. Ocul. Pharmacol. 10(1):307-317

Takahashi, Y., Itami, T. and Kondo, M. (1995) Immunodefense systems of crustacea. Fish Pathol. 30:141-150

Takemori, S., Yamasaki, T., and Ikushiro, S-I (1993) "Cytochrome P-450-linked electron transport system in monooxygenase reaction" in Cytochrome P450. Omura, T.,

Ishimura, Y., and Fujii-Kuriyama, Y. eds. VCH Publishers Inc., New York. pg. 44-63

Tcholakian, R. K., and Eik-Nes, K. B. (1971) Steroidogenesis in the blue crab, *Callinectes sapidus* Rathbun [Rathburn]. Gen. Comp. Endocrinol. 17:115-124

Teshima, S., and Kanazawa, A. (1971) Bioconversion of progesterone by the ovaries of crab, *Portunus trituberulatus*. Gen. Comp. Endocrinol. 17:152-157

Tilly, J.L., Kowalski, K.I., Schomberg, D.W. and Hsueh, A.J. (1992) Apoptosis in atretic ovarian follicles is associated with selective decrease in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 Aromatase. Endocrinology 131:1670-1676

Toda, K., Simpson, E.R., Mendelson, C.R., Shizuta, Y. and Kilgore, M.W. (1994) Expression of the gene encoding Aromatase cytochrome P450 (CYP19) in fetal tissues. Mol. Endocrinol. 8:210-217

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354

Trant, J. M. (1996) Functional expression of recombinant spiny dogfish shark (*Squalus acanthias*) cytochrome P450c17 (17α-hydroxylase/$C_{17,20}$-lyase) in yeast (*Pichia pastoris*). Arch. Biochem. Biophys. 326:8-14

Ueno, T., and Gonzalez, F. J. (1990) Complete sequence of rat CYP2A3 gene specifically transcribed in lung. Nucleic Acids Res. 18:4623-4624

von Moltke, L.L., Greenblatt, D.J., Cotreau-Bibbo, M.M., Duan, S.X., Harmatz, J.S. and Shrader, R.I. (1994) Inhibition of desipramine hydroxylation in vitro by serotonin-reuptake-inhibitor antidepressants, and by quinidine and ketoconazole: a model system to predict drug interaction in vivo. J-Pharmacol-Exp-Ther. 268(3):1278-1283

Warner, M. and Gustafsson, J.A. (1994) Effect of ethanol on cytochrome P450 in the rat brain. Proc. Natl. Acad.Sci. USA 91:1019-1023

Williams, R.T. (1959). Detoxification Mechanisms, Chapman and Hall, London.

Worden, M.K., Kravitz, E.A. and Goy, M.F. (1995) Peptide F-1, an N-terminally extended analog of FMRFamide, enhances contractile activity in multiple target tissues in lobster. J. Exp. Biol. 198: 97-108

Yamano,S., Nhamburo, P.T., Aoyama, T., Myer, U.A., Inaba, T., Kalow, W., Gelboin, H.V., McBride, O.W. and Gonzalez, F.J. (1989) cDNA cloning and sequence and cDNA-directed expression of human P450IIB1: Identification of a normal and two variant cDNAs derived from the CYP2B locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. Biochemistry 28:7340-7348

Yasukochi, Y. and Masters, B.S.S. (1976) Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251(17):5337-5344

Young, N. J., Quinlan, P. T., and Goad, L. J. (1992) Progesterone metabolism *in vitro* in the decapod crustacean, *Penaeus monodon*. Gen. Comp. Endocrin. 87:300-311

BIOGRAPHICAL SKETCH

Sean Michael Boyle was born on the day of January 6, 1966. His father, John Jude Boyle, was just out of medical school and his mother, Donna Deloris Boyle, had been working in the mental ward of a hospital.

He has two older sisters, Melissa Renee and Michelle Davina. Both are married and have one child each, meaning that Sean is in fact an uncle to his nephew Ethan and his niece Emily. He has one younger bother, Christopher David. Sean also has two younger sisters, Kelly Ann and Katie Marie, brought into this world by his stepmother, also Donna Boyle.

Sean was educated in a series of excellent schools in Gainesville, Florida. From the sixth grade to the eighth, he spent rather hot days obtaining a good, solid Catholic education at St. Patricks School. He was taught by strict nuns. From the ninth grade to twelfth, he attended Oak Hall Private School. Sean grew up in Gainesville, Florida. The author lived in Ireland for a short time, from age 9 to 12, and may end his days there.

After five years of painful undergraduate course work at the University of Florida, he finally obtained a degree in zoology. After working for a year or so as a technician, he entered Graduate School in Medicinal Chemistry at the University of Florida. Once there, he did not find course work painful, in fact, he rather enjoyed didactic exercises. After five, or so, years, he graduated with a degree in medicinal chemistry with specialization in toxicology. He now resides in Washington State, pursuing postdoctoral training at the University of Washington.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Margaret O. James, Chair
Professor of Medicinal
Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Raymond Bergeron
Graduate Research
Professor of Medicinal
Chemistry

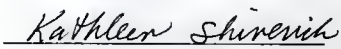
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William Buhi
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

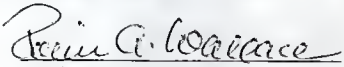
Robert Greenberg
Assistant Scientist of the
Whitney Laboratory

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Kathleen Shiverick
Professor of Pharmacology
and Therapeutics

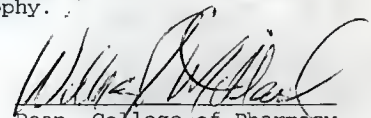
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Robin Wallace
Professor Emeritus of
Anatomy and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1997



Dean, College of Pharmacy

_____

Dean, Graduate School